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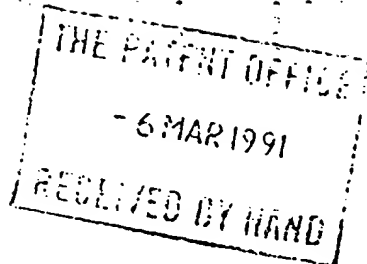
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BINDING SUBSTANCES

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BINDING SUBSTANCES

The present invention relates to binding substances. The present invention also relates to methods for the production of binding substances eg binding molecules and to the biological binding molecules produced by these methods. The present invention also relates to: a) the production of antibodies, receptor molecules and fragments and derivatives of these antibodies and receptor molecules; b) viruses encoding the above identified molecules, which viruses have the ability to present said molecules at their surfaces; c) packages comprising a virus and an above identified molecule presented at the viral surface; and d) screening techniques utilising the unique properties of these packages.

Owing to their high specificity for a given antigen, the advent of monoclonal antibodies (Kohler, G. and Milstein C; 1975 Nature 256: 495) represented a significant technical break-through with important consequences both scientifically and commercially.

Monoclonal antibodies are made by establishing an immortal mammalian cell line which is derived from a single immunoglobulin producing cell secreting one form of a biologically functional antibody molecule with a particular specificity. Because the antibody-secreting mammalian cell line is immortal, the characteristics of the antibody are reproducible from batch to batch. The key properties of monoclonal antibodies are their specificity for a particular antigen and the reproducibility with which they can be manufactured.

Structurally, the simplest antibody (IgG) comprises four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulphide bonds (see figure 1). Each chain has a constant region (C) and a variable region (V). The antibody has two arms (the Fab region) each of which has a V_L and a V_H region associated with each other. It is this pair of V regions (V_L and V_H) that differ

from one antibody to another, and which together are responsible for recognising the antigen. In even more detail, each V region is made up from three complementarity determining regions (CDR) separated by four framework regions (FR). The CDR's are the most variable part of the variable regions, and they perform the critical antigen binding function. The CDR regions are derived from many potential germ line sequences via a complex process involving recombination, mutation and selection.

It has been shown that the function of binding antigens can be performed by fragments of a whole antibody. Binding fragments are the F_V fragment which comprises the V_L and V_H of a single arm of the antibody, and the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989); which consists of a single heavy chain variable domain (V_H).

Although the F_V fragment is coded for by separate genes, it has proved possible to construct a linker that enables them to be made as a single protein chain (known as single chain F_V (scFv); Bird, R.E. et al., Science 423, 423-426 (1988) Huston, J.S. et al., Proc. Natl. Acad. Sci., USA 85, 5879-5883 (1988)) by recombinant methods.

Whilst monoclonal antibodies, their fragments and derivatives have been enormously advantageous, there are nevertheless a number of limitations associated with them.

Firstly, the therapeutic applications of monoclonal antibodies produced by human immortal cell lines holds great promise for the treatment of a wide range of diseases (Clinical Applications of Monoclonal Antibodies. Edited by E. S. Lennox. British Medical Bulletin 1984. Publishers Churchill Livingstone). Unfortunately, immortal antibody-producing human cell lines are very difficult to establish and they give low yields of antibody (approximately 1 $\mu\text{g/ml}$). In contrast, equivalent rodent cell lines yield high amounts of antibody (approximately 100 $\mu\text{g/ml}$). However, the repeated administration of these foreign rodent proteins to humans can lead to harmful hypersensitivity reactions. In the main therefore, these

rodent-derived monoclonal antibodies have limited therapeutic use.

Secondly, a key aspect in the isolation of monoclonal antibodies is how many different antibody producing cells with different specificities, can be sampled, compared to how many need to be sampled in order to isolate a cell producing antibody with the desired specificity characteristics (Milstein, C., Royal Soc. Croonian Lecture, Proc. R. Soc. London B. 239; 1-16, (1990)). For example, the number of different specificities expressed at any one time by lymphocytes of the murine immune system is thought to be approximately 10^7 and this is only a small proportion of the potential repertoire of specificities. However, during the isolation of a typical antibody producing cell with a desired specificity, the investigator is only able to sample 10^3 to 10^4 individual specificities. The problem is worse in the human, where one has approximately 10^{12} lymphocyte specificities, with the limitation on sampling of 10^3 or 10^4 remaining.

This problem has been alleviated to some extent in laboratory animals by the use of immunisation regimes. Thus, where one wants to produce monoclonal antibodies having a specificity against a particular epitope, an animal is immunised with an immunogen expressing that epitope. The animal will then mount an immune response against the immunogen and there will be a proliferation of lymphocytes which have specificity against the epitope. Owing to this proliferation of lymphocytes with the desired specificity, it becomes easier to detect them in the sampling procedure. However, this approach is not successful in all cases, as a suitable immunogen may not be available. Furthermore, where one wants to produce human monoclonal antibodies (eg for therapeutic administration as previously discussed) such an approach is not practically or ethically feasible.

In the last few years, these problems have in part,

been addressed by the application of recombinant DNA methods to the isolation and production of antigen binding fragments of an antibody molecule in bacteria such as E.coli. Furthermore, the use of polymerase chain reaction (PCR) amplification (Saiki, R.K., et al., Science 239, 4387-491 (1988)) to isolate antibody producing sequences from cells and organs, has great potential for speeding up the timescale under which specificities can be isolated. Amplified V_H and V_L genes are cloned directly into vectors for expression in bacteria or mammalian cells (Orlandi, R., et al., 1989, Proc. Natl. Acad. Sci., USA 86, 3833-3837; Ward, E.S., et al., 1989 supra; Larrick, J.W., et al., 1989, Biochem. Biophys. Res. Commun. 160, 1250-1255; Sastry, L. et al., 1989, Proc. Natl. Acad. Sci., USA., 86, 5728-5732). Conversely, some of these techniques can exacerbate the screening problems. For example, large separate heavy and light chain libraries have been produced from immunized mice and combined together in a random combinatorial manner prior to screening (Huse, W.D. et al., 1989, Science 246, 1275-1281). Crucially however, the information held within each cell, namely the specific combination of one light chain with one heavy chain, is lost. This loses most, if not all, of the advantage gained by using immunization protocols in the animal. Currently, only libraries derived from single heavy chain variable domains (dAbs; Ward, E.S., et al., 1989, supra.) do not suffer this drawback, but because not all antibody heavy chain variable regions are capable of binding antigen, more have to be screened.

In addition, the problem of directly screening many different specificities in prokaryotes remains to be solved.

Thus, there is a need for a screening system which ameliorates or overcome one or more of the above or other problems. The ideal system would allow the sampling of very large numbers of specificities (eg of the order of 10^6 and higher) rapid sorting at each cloning round, and rapid

transfer of the genetic material coding for the binding molecule from one stage of the production process, to the next stage.

5 The most attractive candidates for this type of screening, would be prokaryotic organisms (because they grow quickly, are relatively simple to manipulate and because large numbers of clones can be created) which express and retain antibody on their surface. It has already been shown that antibody fragments can be secreted through bacterial
10 membranes with the appropriate signal peptide (Skerra, A., and Pluckthun, A., 1988, Science 240, 1038-1040; Better, M. et al., 1988, Science 240, 1041-1043). However, it has not been shown how an antibody or antibody fragment can be held on the bacterial cell surface in a configuration which
15 allows efficient sampling of its antigen binding properties. In large part, this is because the bacterial surface is a complex structure, and in the gram-negative organisms there is an outer wall which further complicates the position.

Bacteriophage make attractive candidates because in
20 general their surface is a much simpler structure, they can be grown easily in large numbers, are amenable to the practical handling involved in many potential mass screening programmes and they carry genetic information for their own synthesis within a small, simple package. The difficulty
25 has been to practically solve the problem of how to use bacteriophages in this manner. For example, a Genex Corporation patent application number PCT/US88/00716 has proposed that the bacteriophage lambda would be a suitable vehicle for the expression of antibody molecules, but no
30 proposals provide a teaching which enables the general idea to be carried out. For example PCT/US88/00716 does not demonstrate that any sequences: a) have been expressed as a fusion with gene V; b) have been expressed on the surface of lambda; and c) retain biological activity. Furthermore
35 there is no teaching on how to screen for suitable fusions.

The problem of how to use bacteriophages is in fact a

difficult one. The antibody molecule must be inserted into the phage in such a way that the integrity of the phage coat is not undermined, and the antibody itself should be biologically active. Thus the antibody should fold efficiently and correctly and be presented for antigen binding. However, solving the problem for antibody molecules and fragments would also provide a general method for the screening of many receptor molecules.

Surprisingly, the applicants have been able to construct a bacteriophage that expresses and presents on its surface large binding molecules (eg large biologically functional antibody molecules) and which remains intact and infectious. The applicants have called the structure which comprises a virus particle and a binding molecule presented at the viral surface a 'package'. Where the binding molecule is an antibody (or a fragment or derivative of an antibody), the applicants call the package a phage antibody. However, except where the context demands otherwise, where the term phage antibody is used generally it should also be interpreted as referring to any package comprising a virus particle and a binding molecule presented at the viral surface. Phage-antibodies (pAbs) are likely to find a range of applications in screening antibody V-genes encoding antigen binding activities. For example, pAbs could be used in cloning and rescue of hybridomas (Orlandi, R., et al. (1989) PNAS 86 p3833-3837), and in the screening of large combinatorial libraries (such as found in Huse, W.D. et al., 1989, Science 246, 1275-1281). In particular, rounds of selection using pAbs may help in rescuing the higher affinity antibodies from the latter libraries. It may be preferable to screen small libraries derived from antigen-selected cells (Casali, P., et al., (1986) Science 234 p476-479) to rescue the original VH/VL pairs comprising the V region of an antibody. The use of pAbs may also allow the construction of entirely synthetic antibodies. For example, V-gene repertoires could be made in vitro by combining unrearranged V genes, with D and J segments. Libraries of

pAbs could then be selected by binding to antigen, hypermutated in the antigen-binding loops in vitro and subjected to further rounds of selection and mutagenesis.

5 The demonstration that a functional antigen-binding site can be expressed on the surface of phage, has implications beyond the construction of novel antibodies. For example, if other protein domains can be expressed at the surface of a phage, phage vectors could be used to clone and select genes by the binding properties of the expressed
10 protein. Furthermore, endless variants of proteins, including epitope libraries built into the surface of the protein, could be made and readily selected for binding activities. In effect other protein architectures might serve as "nouvelle" antibodies. One class of molecules that
15 could be useful in this type of application are receptors. For example, a specific receptor could be expressed on the surface of the phage such that it would bind its ligand. The receptor could then be modified by, for example, in vitro mutagenesis and variants having higher binding
20 affinity for the ligand selected. The screening may be carried out according to one or more of the formats described below with reference to figure 2. Figure 2 refers particularly to phage antibodies. In the situation discussed above the phage antibody is replaced with a phage
25 receptor and the antigen with a ligand 1.

Alternatively, the receptor-phage could be used as the basis of a rapid screening system for the binding of ligands, altered ligands, or potential drug candidates. The advantages of this system of simple cloning, convenient
30 expression, standard reagents and easy handling makes the drug screening application particularly attractive. In the context of this discussion, receptor means a molecule that binds a specific, or group of specific, ligand(s); the natural receptor could be expressed on the surface of a
35 population of cells, it could be the extracellular domain of such a molecule (whether such a form exists naturally or not), or it could be a soluble molecule performing a natural

binding function in the plasma or within a cell or organ.

Although throughout this application the applicant discuss the possibility of screening for higher affinity variants of phage-antibodies, they recognise that in some applications, for example low affinity chromatography (Ohlson, S. et al Anal. Biochem. 169, p204-208 (1988)), it may be desirable to isolate lower affinity variants.

The system of expressing binding molecules on the phage surface could also be used as a primary cloning system. For example, a cDNA library could be constructed and inserted into the bacteriophage and this library screened for the ability to bind a ligand. The ligand/binding molecule combination could include receptor/ligand, enzyme/substrate (or analogue), nucleic acid binding protein/nucleic acid etc. This could be a preferred method to isolate a clone of either molecule in the pair, if the other molecule was available.

Targeted gene transfer

A useful and novel set of applications makes use of the binding protein on the phage to target the phage genome to a particular cell or group of cells. For example, a phage-antibody specific for a cell surface receptor could be used to bind to the target cell surface. The phage could then be internalised, either through the action of the receptor itself or as the result of another event (eg. an electrical discharge such as in the technique of electroporation). The phage genome would then be expressed if the relevant control signals (for transcription and translation and possibly replication) were present. This would be particularly useful if the genome contained a sequence whose expression was desired in the target cell (along with the appropriate expression control sequences). A useful sequence might confer antibiotic resistance to the recipient cell or label the cell by the expression of its product (eg. if the sequence expressed a detectable gene product such as a

luciferase, see White, M, et al, Techniques 2(4), p194-201 (1990)), or confer a particular property on the target cell (eg. if the target cell was a tumour cell and the new sequence directed the expression of a tumour suppressing gene), or express an antisense construct designed to turn off a gene or set of genes in the target cell, or express a gene or gene product designed to be toxic to the target cell.

This technique of "targeted gene transfer" has a number of uses in research and also in therapy and diagnostics. For example, gene therapy often aims to target the replacement gene to a specific cell type that is deficient in its activity; targeted pAbs provide an answer to this problem. In diagnostics, phage specific for particular bacteria or groups of bacteria have been used to target marker genes, eg. luciferase, to the bacterial host (see, for example, Ulitzer, S., and Kuhn, J., EPA 85303913.9). If the host range of the phage is appropriate, only those bacteria that are being tested for, will be infected by the phage, express the luciferase gene and be detected by the light they emit. This system has been used to detect the presence of Salmonella. One major problem with this approach is the initial isolation of a bacteriophage with the correct host range and then cloning a luciferase gene cassette into that phage, such that it is functional. The pAb system allows the luciferase cassette to be cloned into a well characterised system (filamentous phage) and allows simple selection of an appropriate host range, by modifying the antibody (or other binding molecule) specificity the pAb contains.

The applicant has also shown that enzymes can be expressed on the phage surface. Useful applications of this invention include the cloning of enzyme coding genes, or the design and selection of mutant enzymes with enhanced properties on particular substrates. For example, conditions can be used whereby the enzyme (or modified enzyme) binds a particular substrate, product

intermediate (or analogues of them) to identify phage from a library containing a desired activity or by subjecting phage already expressing the enzyme, to in vitro mutagenesis, followed by selection of those variants with a desired level of binding and/or catalysis.

The present applicants have also been able to develop novel screening systems and assay formats which depend on the unique properties of these packages eg phage antibodies.

The present invention provides a method for producing a package which method comprises the steps of:

- a) inserting a nucleotide sequence encoding the binding molecule within a viral genome;
- b) culturing the virus containing said nucleotide sequence, so that said binding molecule is expressed by the virus presented at its surface.

The present invention also provides a method for producing a binding molecule specific for a particular epitope which comprises producing a package as describe above and the additional step of screening for said binding molecule by binding of said molecule to said epitope. The method may comprise one or more of the additional steps of: i) separating the package from the epitope; ii) recovering said package; and iii) using the inserted nucleotide sequence in a recombinant system to produce the binding molecule separate from virus. The screening step may isolate the nucleotide sequence encoding the binding molecule of desired specificity, by virtue of said binding molecule being expressed in association with the surface of the virus.

In the above methods, the binding molecule may be an antibody, or a fragment or derivative of an antibody. Alternatively, the binding molecule may be an enzyme or receptor and fragments/derivatives of any such enzymes or receptors.

In the above methods, the virus may be a filamentous F-specific bacteriophage. The filamentous F-specific bacteriophage may be fd. In particular, it may be a

tetracycline resistant version of fd known as fd-tet. The nucleotide sequence may be inserted within the gene III region of fd. The sequence may be inserted after the signal sequence of gene III, preferably after amino acid+1 of the mature protein. The site for insertion may be flanked by short sequences corresponding to sequences which occur at each end of the DNA to be inserted. For example, the protein domain is an immunoglobulin domain, the insertion site in the phage may be flanked by nucleotide sequences which code for the first five amino acids and the last five amino acids of the Ig domain. Such flanking nucleotide sequences are shown in figure 4(2) B and C, wherein the site-flanking nucleotide sequences encode amino acid sequences QVQLQ and VTVSS which occur at either end of the V_H domain, or QVQLQ and LEIKR which occur at either end of the F_V (combined $V_H + V_L$) domain. Each of these sequences flanking the insertion site may include a suitable cleavage site, as shown in Fig 4.

Alternatively, the flanking nucleotide sequences shown in figure 4(2) B and C as described above, may be used to flank the insertion site for any DNA to be inserted, whether or not that DNA codes an immunoglobulin.

In the above methods the nucleotide sequences inserted within the viral genome may be derived from eg mammalian spleen cells or peripheral blood lymphocytes. The mammal may be immunised or non-immunised. Alternatively, the nucleotide sequence may be derived by the in vitro mutagenesis of an existing antibody coding sequence. The phage particle presenting said binding molecule may remain intact and infectious.

As previously mentioned, the present invention also provides novel screening systems and assay formats. In these systems and formats the gene sequence encoding the binding molecule (eg the antibody) of desired specificity is separated from the general population having a range of specificities by the fact of its binding to a specific target (eg the antigen or epitope).

Thus, the present invention provides a method of screening a population of phage antibodies (where the binding molecule is an antibody) for a phage antibody with a desired specificity, which comprises contacting said population of phage antibodies with a desired epitope and separating phage antibody which binds to said epitope, from said epitope. The means for separating any binding phage antibodies may be varied in order to obtain binding phage antibodies with different binding affinities for said epitope.

Alternatively, in order to obtain high affinity phage antibodies the epitope may be presented to the population of phage antibodies already with a binding member for said epitope bound thereto, in which case, phage antibodies with a higher binding affinity for said epitope than said bound binding member will displace said bound binding member. The high affinity phage antibodies can then be separated from said epitope.

Separation of phage antibodies from said epitope may be achieved by eg elution techniques well known in the art, infection of suitable bacteria etc.

The present invention also provides packages as defined above and binding molecules (eg antibodies, enzymes, receptors; fragments and derivatives thereof), obtainable by use of any of the above defined methods, systems and formats.

The applicants have chosen the filamentous F- specific bacteriophages as an example of the type of phage that could provide a vehicle for the expression of antibodies and antibody fragments and derivatives on their surface and facilitate subsequent screening and manipulation.

The F-specific phages (eg fl, fd and M13) have evolved a method of propagation which does not kill the host cell and they are used commonly as vehicles for recombinant DNA (Kornberg, A., DNA Replication, W.H. Freeman and Co., San Francisco, 1980). The single stranded DNA genome (approximately 6.4 Kb) is extruded through the bacterial

membrane where it sequesters capsid sub-units, to produce mature virions. These virions are 6 nm in diameter, 1µm in length and each contain approximately 2,800 molecules of the major coat protein encoded by viral gene VIII and four
 5 molecules of the adsorption molecule encoded by viral gene III. The latter is located at one end of the virion. The structure has been reviewed by Webster et al., 1978 in *The Single Stranded DNA Phages*, 557-569, Cold Spring Harbor Laboratory Press. The gene III product is involved in the
 10 binding of the phage to the bacterial F-pilus.

Although these phages do not kill their host during normal replication, disruption of some of their genes can lead to cell death (Kornberg, A., 1980 supra.) This places some restraint on their use. The applicants have recognized
 15 that gene III of phage fd is an attractive possibility for the insertion of biologically active foreign sequences. The protein itself is only a minor component of the phage coat and disruption of the gene does not lead to cell death (Smith, G. 1988, *Virology* 167: 156-165). Furthermore, it is
 20 possible to insert some foreign sequences (with no biological function) into various positions within this gene (Smith, G., 1985 *Science* 228: 1315-1317., Parmley, S.F. and Smith, G.P *Gene*: 73 (1988) p. 305-318., and de la Cruz, V.F., et al., 1988, *J. Biol. Chem.*, 263: 4318-4322). In
 25 these cases, although the infectivity of the virion was disrupted, the inserted sequences could be detected on the phage surface.

The protein encoded by gene III has several domains (Pratt, D., et al., 1969 *Virology* 39:42-53., Grant, R.A., et
 30 al., 1981, *J. Biol. Chem.* 256: 539-546 and Armstrong, J., et al., *FEBS Lett.* 135: 167-172 1981.) including: i) a signal sequence that directs the protein to the cell membrane and which is then cleaved off; ii) a domain that anchors the mature protein into the bacterial cell membrane (and also
 35 the phage coat); and iii) a domain that specifically binds to the phage receptor the F-pilus of the host bacterium. Short sequences derived from protein molecules have been

inserted into two places within the mature molecule (Smith, G., 1985 supra., and Parmley, S.F. and Smith, G.P., 1988 supra.) into an inter-domain region and also between amino acids 2 and 3 at the N-terminus. The insertion sites at the N-terminus were more successful in maintaining the structural integrity of the gene III protein and displaying the peptides on the surface of the phage. By use of specific antisera, the peptides inserted into this position were shown to be on the surface of the phage. These authors were also able to purify the phage using this property. However, the peptides expressed by the phage, did not possess measurable biological functions of their own.

Retaining the biological function of a molecule when it is expressed in a radically different context to its natural state is difficult. The demands on the structure of the molecule are heavy. In contrast, retaining the ability to be bound by specific antisera is a passive process which imposes far less rigorous demands on the structure of the molecule. For example, it is the rule rather than the exception that polyclonal antisera will recognise totally denatured, and biologically inactive, proteins on Western blots (see for example, Harlow, E. and Lane, D., Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory Press 1988). Therefore, the insertion of peptides into a region that allows their structure to be probed with antisera teaches only that the region allows the inserted sequences to be exposed and does not teach that the region is suitable for the insertion of large sequences with demanding biological function.

This experience with Western blots is a graphic practical demonstration which shows that retaining the ability to be bound by specific antisera imposes far less rigorous demands on the structure of a molecule, than does the retention of a biological function.

The applicants have investigated the possibility of inserting biologically active antibody fragments into the gene III region of fd to create a large fusion protein.

is apparent from the previous discussion, this approach makes onerous demands on the functionality of the fusion protein. The insertion is large, 100-200 amino acids; the antibody derived domain must fold efficiently and correctly to retain antigen-binding; and most of the functions of gene III must be retained. The applicants approach to the construction of the fusion molecule was designed to minimise the risk of disrupting these functions. The initial vector used was fd-tet (Zacher, A.N., et al., 1980, Gene 9, 127-140) a tetracycline resistant version of fd bacteriophage that can be propagated as a plasmid that confers tetracycline resistance to the infected E.coli host. The applicants chose to insert after the signal sequence of the fd gene III protein for several reasons. In particular, the applicants chose to insert after amino acid 1 of the mature protein to retain the context for the signal peptidase cleavage. To retain the structure and function of gene III itself, the majority of the original amino acids are synthesized after the inserted immunoglobulin sequences. The inserted immunoglobulin sequences were designed to include residues from the switch region that links V_H-V_L to C_H1-C_L . (Lesk, A., and Chothia, C., Nature 335, 188-190, 1988).

Surprisingly, by manipulating gene III of bacteriophage fd, the present applicants have been able to construct a bacteriophage that expresses on its surface large biologically functional antibody molecules and which remains intact and infectious. Furthermore, the phages bearing antibodies of the correct specificity, can be selected from a background where the majority of phages do not show this specificity.

The population of antibody molecules inserted into the phage can be derived from a variety of sources. For example, immunised or non-immunised rodents or humans, and from organs such as spleen and peripheral blood lymphocytes. The coding sequences are derived from these sources by techniques familiar to those skilled in the art (Orlandi,

R., et al., 1989 supra; Larrick, J.W., et al., 1989 supra; Chiang, Y.L., et al., 1989 Bio Techniques 7, p. 360-366; Ward, E.S, et al., 1989 supra; Sastry, L., et al., 1989 supra.) Each individual phage antibody in the
5 resulting library of phage antibodies will express antibody derived fragments that are monoclonal with respect to its antigen-binding characteristics.

The disclosure made by the present applicants is important and provides a significant breakthrough in the
10 technology relating to the production of biological binding molecules, their fragments and derivatives by the use of recombinant methods.

In standard recombinant techniques for the production of antibodies, an expression vector containing sequences
15 coding for the antibody polypeptide chains is used to transform eg E.coli. The antibody polypeptides are expressed and detected by use of standard screening systems.. When the screen detects an antibody polypeptide of the desired specificity, one has to return to the particular
20 transformed E.coli expressing the desired antibody polypeptide. Furthermore, the vector containing the coding sequence for the desired antibody polypeptide then has to be isolated for use from E.coli in the further processing steps.

25 In the present invention however, the desired antibody polypeptide when expressed, is already packaged with its gene coding sequence. This means that when the screen detects an antibody polypeptide of desired specificity, there is no need to return to the original culture for
30 isolation of that sequence.

Because the phage antibody is a novel structure that contains an antibody of monoclonal antigen-binding specificity on the surface of a relatively simple structure also containing the genetic information encoding its
35 function, phage antibodies that bind antigen can be recovered very efficiently by either eluting off (eg using diethylamine, high salt etc) and infecting suitable

bacterial or by denaturing the structure and specifically amplifying the antibody encoding sequences using PCR. That is, there is no necessity to refer back to the original bacterial clone that gave rise to the phage antibody.

5 Individual phage antibodies expressing the desired antigen-binding specificity can be isolated from the complex library using the conventional screening techniques (eg as described in Harlow, E., and Lane, D., 1988, *supra*). One example is illustrated in figure 2(i). This shows antigen
10 (ag) bound to a solid surface (s). The population of phage antibodies is then passed over the antigen, and those individuals p that bind are retained after washing, and optionally detected with detection system d. One possible detection system based upon anti-fd antisera is illustrated
15 below in example 4. Since the bound phage antibody can be amplified using for example PCR or bacterial infection, it is also possible to rescue the desired specificity even when insufficient individuals are bound to allow detection via conventional techniques.

20 The efficiency of this screening procedure for phage antibodies and the ability to create very large libraries means that the immunisation techniques developed to increase the proportion of screened cells producing antibodies of interest will not be an absolute requirement. The technique
25 allows the rapid isolation of antigen-binding specificities, including those that would be difficult or even unobtainable by conventional techniques, for example, catalytic or anti-idiotypic antibodies. Removal of the animal altogether is now possible once a complete library of the immune
30 repertoire has been constructed.

Affinity Maturation Screening Formats

The applicants have also devised a series of novel screening techniques that are practicable only because of the unique properties of phage antibodies. The general
35 outline of some screening procedures is illustrated in figure 2.

The population/library of phage antibodies to be

screened could be generated from immunised or other animals; or be created in vitro by mutagenising pre-existing phageantibodies (using techniques well-known in the art such as oligonucleotide directed mutagenesis (Sambrook, J., et al., 1989 Molecular Cloning a Laboratory Manual, Cold Spring Harbor Laboratory Press). This population can be screened in one or more of the formats described below with reference to figure 2, to derive those individual phage antibodies whose antigen binding properties are different from sample c. Examples of the possible screening formats are:

Binding/Elution

Referring to figure 2(i) population p binds to antigen ag fixed to a solid support s. If samples of bound population p are removed under increasingly stringent conditions, the binding affinity represented in each sample will increase. Conditions of increased stringency can be obtained, for example, by increasing the time of soaking or changing the pH of the soak solution, etc.

Competition

Referring to figure 2(ii) antigen ag can be bound to a solid support s and bound to saturation by the original binding molecule c. If a population of mutant phage antibody (or a set of unrelated phage antibody) p is offered to the complex, only those that have higher affinity for antigen ag than c will bind. In most examples, only a minority of population c will be displaced by individuals from population p. If c is a traditional antibody molecule, all bound material can be recovered and bound p recovered by infecting suitable bacteria and/or by use of standard techniques such as PCR.

An advantageous application is where ag is used as a receptor and c the corresponding ligand. The recovered bound population p is then related structurally to the receptor binding site/and or ligand. This type of specificity is known to be very useful in the pharmaceutical

industry.

Another advantageous application is where ag is an antibody and c its antigen. The recovered bound population p is then an anti-idiotypic antibody which have numerous uses in research and the diagnostic and pharmaceutical industries.

In some instances it may prove advantageous to pre-select population p . For example, in the anti-idiotypic example above, p can be absorbed against a related antibody that does not bind the antigen.

However, if c is a phage antibody, then either or both c and p can advantageously be marked in some way to both distinguish and select for bound p over bound c . This marking can be physical, for example, by pre-labelling p with biotin; or more advantageously, genetic. For example, c can be marked with an $EcoB$ restriction site, whilst p can be marked with an $EcoK$ restriction site (see Carter, P. et al., 1985, Nucl. Acids Res. 13, 4431-4443). When bound $p+c$ are eluted from the antigen and used to infect bacteria, there is restriction (and thus no growth) of population c (i.e. $EcoB$ restricting bacteria in this example). Any phage that grew, would be greatly enriched for those individuals from p with higher binding affinities. Alternatively, the genetic marking can be achieved by marking p with new sequences, which can be used to specifically amplify p from the mixture using PCR.

The novel structure of the phage antibody molecule can be used in a number of other applications some examples of which are:

Signal Amplification

Acting as a novel molecular entity in itself, phage antibodies combine the ability to bind the specific antigen with the amplification, if the major coat protein is used to attach another moiety. This moiety can be attached via immunological, chemical, or any other means and can be used,

for example, to label the complex with detection reagents or cytotoxic molecules for use in vivo or in vitro.

Physical Detection

The size of the phage antibody can be used as a marker particularly with respect to physical methods of detection such as electron microscopy and/or some biosensors, eg. surface plasmon resonance.

Diagnostic Assays

The phage antibody molecule also has advantageous uses in diagnostic assays, particularly where separation can be effected using its physical properties for example centrifugation, filtration etc.

In order that the invention is more fully understood, embodiments will now be described in more detail by way of example only and not by way of limitation with reference to the figures described below.

Figure 1 shows the basic structure of the simple antibody molecule IgG.

Figure 2 shows schematically novel screening techniques which utilise the unique properties of phage antibodies.

Figure 3 shows a scheme for the construction of vectors.

Figure 4 shows the nucleotide sequences for the oligonucleotides and vectors. All sequences are drawn 5' to 3' and are numbered according to Beck et al., 1978, Nucl. Acid Res., 5: 4495-4503. 4.1 shows the sequences of the oligonucleotides used for mutagenesis (oligo's 1 and 2) or sequencing (oligo 3). The sequences shown were synthesized on an Applied Biosystems, oligonucleotide synthesizer and are complementary to the single stranded form of fd-tet (they are in the anti-sense form with respect to gene III). 4.2 shows the sequences of the various constructs around the gene III insertion site. these sequences are drawn in the sense orientation with respect to gene III; a) fd-tet (and

FDTdBst) b) FDTPs/Bs and c) FDTPs/Xh. The key restriction enzyme sites are shown along with the immunoglobulin amino acids contributed by the vectors, (amino acid single letter code is used, see Harlow, E., and Lane, D., 1988 supra.).

Figure 5 shows the nucleotide and amino acid sequences for scFv in the vector scFvD1.3 myc. This gives the sequence of the anti-lysozyme single chain Fv and surrounding sequences in scFvD1.3 myc showing the N-terminal pel B signal peptide sequence and the C-terminal myc tag sequence (Ward, E.S., et al., 1989, supra.). Also highlighted is the peptide sequence linking the V_H and V_L regions. The amino acid sequence is represented above the nucleotide sequence by the single letter code, see Harlow, E., and Lane, D., 1988 supra..

Figure 6 shows the effect of varying the amount of supernatant on the binding of phage antibodies to lysozyme in graphical form. Each point is the average of duplicate samples. Lysozyme was coated at 1 mg/ml in 50 mM NaHCO_3 .

Figure 7 shows the effect of varying the coating concentration on the binding of phage antibodies to lysozyme in graphical form. Each point is the average of duplicate samples. Lysozyme was coated with the specified concentration of either BSA or lysozyme.

Figure 8 shows the sequence around the cloning site in gene III of fd-Cat2.

Figure 9 shows the binding of pAb (D1.3) to lysozymes. Binding of phage as detected by ELISA to (a) hen egg-white lysozyme (HEL) (b) turkey egg-white lysozyme (TEL), (c) human lysozyme (HUL), (d) bovine serum albumin (BSA). A further control of (c) fd-CAT1 to HEL.

Figure 10 shows a map of fab D1.3 in pUC19.

Figure 11 shows the ELISA results providing a comparison of lysozyme-binding by phage-Fab and phage-ScFv. Vector=fd-CAT2 (example 5); fdSCFV(OX)=pAbNQ11 (Example 9); fdVHCH1(D1.3)=grown in normal cells (ie. no light chain, see

example 7); fdfab-fdfab-fdVHCH1 (D1.3) grown in cells containing D1.3 light chain; fdSCFV (D1.3)=pAbD1.3.

Figure 12 shows oligonucleotide probing of affinity purified phage. 10^{12} phage in the ratio 1 pAb (D1.3) in
5 4×10^4 fd-CAT1 phages were affinity purified and probed with an oligonucleotide specific for pAb (D1.3) (a) filter after one round of affinity purification (900 colonies total) and, (b) after two rounds (372 colonies total).

10 Figure 13 shows the sequence of the anti-oxazalone antibody NQ11.

Figure 14 shows the ELISA results for binding of pAb NQ11 and pAb D1.3 and Vector FDTPs/xh to specified antigens.

15 Figure 15 shows the sequence surrounding phoA insertion in fd-phoA1. The restriction sites used for cloning are shown, as well as the amino acids encoded by phoA around the insertion site. The first five amino acids of the mature fusion come from gene 3.

20 Figure 16(1) shows the structure of gene 3 and figure 16(2) shows the peptide linker sites A and B.

Figure 17 shows schematically the protocol for PCR assembly of mouse VH and VLK repertoires for phage display described in example 16.

25 Figure 18 shows examples of the final products obtained with the procedure of example 16. Lanes a and b

show the products of the initial PCR using heavy and light chain primers respectively; lane c shows the complete assembled 700bp product before final digestion with NotI and ApalI; M1, M2 markers Φ 174 Hae III digest and 123 base pair ladder (BRL Limited, P.O. Box 35, Washington Road, Paisley, Scotland) respectively.

Figure 19 shows the binding of ^{125}I -PDGF-BB to fd h-PDGFB-R phage in immunoprecipitation assay and comparison to fd TPs/Bs and no phage controls; binding is expressed as a percentage of the total ^{125}I -PDGF-BB added to the incubation.

Figure 20 shows the ~~displacement~~ *competitive reduction* of ^{125}I -PDGF-BB bound to fd-h-PDGFB-R phage using unlabelled PDGF-BB measured using an immunoprecipitation assay. Binding is expressed as a percentage of the total ^{125}I -PDGF-BB added to the incubation.

Figure 21 shows the ~~displacement~~ *competitive reduction* of ^{125}I -PDGF-BB bound to fd-h-PDGFB-R phage using unlabelled PDGF-BB measured using an immunoprecipitation assay. Non-specific binding of ^{125}I -PDGF-BB to vector phage fdTPs/Bs in the absence of added unlabelled PDGF was deducted from each point.

Figure 22 shows the results of an ELISA of lysozyme binding by pCAT-3 ScFv D1.3 phagemid in comparison with pCAT-3 vector (both rescued by M13KO7) and fdCAT2 ScFv D1.3 as described in example 17. The ELISA was performed

as described in example 6 with modifications detailed in example 17.

The following procedures used by the present applicants are described in Sambrook, J. et al., 1989, supra.: restriction digestion, ligation, preparation of competent cells (Hanahan method), transformation, analysis of restriction enzyme digestion products on agarose gels, purification of DNA using phenol/chloroform, 5'-end labelling of oligonucleotides, filter screening of bacterial colonies, preparation of 2xTY medium and plates, preparation of tetracycline stock solutions, PAGE of proteins, preparation of phosphate buffered saline.

All enzymes were supplied by New England Biolabs (CP Laboratories, PO Box 22, Bishop's Stortford, Herts., England) and were used according to manufacturer's instructions unless otherwise stated.

The vector fd-tet (Zacher, A.N. et al., 1980, supra) was obtained from the American Type Culture Collection (ATCC

No. 37000) and transformed into competent TG1 cells (genotype: K126 (lac-pro), sup E, thi, hsdD5/F'traD36, pro A+B+, Lac I^q, lac δ M15).

Viral particles were prepared by growing TG1 cells containing the desired construct in 10 to 100 mls 2xTY medium with 15 μ g/ml tetracycline for 16-24 hours. The culture supernatant was collected by centrifugation for 10 mins at 10,000 rpm in an 8 x 50 ml rotor, Sorval RC-5B centrifuge. Phage particles were precipitated by adding 1/5th volume 20% polyethylene glycol (PEG)/2.5M NaCl and leaving at 4°C for 1 hour. These were spun for 15 minutes as described above and the pellets resuspended in 10 mM Tris/HCl pH 8, 1 mM EDTA to 1/100th of the original volume. Residual bacteria and undissolved material were removed by spinning for 2 minutes in a microcentrifuge. Single stranded DNA for mutagenesis or sequencing was prepared from concentrated phage according to Sambrook, J., et al., 1989, supra.

Example 1

Design of Insertion Point Linkers and Construction of Vectors

The vector fd-tet has two BstEII restriction sites flanking the tetracycline resistance gene (fig 3). Since the strategy for inserting the V_H fragments was to ligate them into a newly inserted BstEII site within gene III, it was advantageous to delete the original BstEII sites from fd-tet. This was achieved by digesting fd-tet with the restriction enzyme BstEII, filling-in the 5' overhangs and re-ligating to generate the vector FDT6Bst. Digestion of fd-tet with BstEII (0.5 units/ μ l) was carried out in 1x KGB buffer (100 mM potassium glutamate, 25 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 μ g/ml bovine serum albumin, 0.5 mM (Sambrook, J., et al., 1989, supra.) with DNA at a concentration of 25 ng/ μ l. The 5' overhang was filled in, using 2x KGB buffer, 250 μ M each dNTP's

(Pharmacia Ltd., Pharmacia House, Midsummer Boulevard, Milton Keynes, Bucks., UK.) and Klenow Fragment (Amersham International, Lincoln Place, Green End, Aylesbury, Bucks., UK) at 0.04 units/ μ l. After incubating for 1 hour at room temperature, DNA was extracted with phenol/chloroform and precipitated with ethanol.

Ligations were carried out at a DNA concentration of 50ng/ μ l for 1 hour at room temperature using T4 DNA ligase (40 units/ μ l). Ligations were transformed into competent TG1 cells and plated onto TY plates supplemented with 15 μ g/ml tetracycline. Colonies were picked into 25 mls of 2xTY medium supplemented with 15 μ g/ml tetracycline and grown overnight at 37°C.

Double stranded DNA was purified from the resulting clones using the gene-clean II kit (Biol01 Inc., PO Box 2284, La Jolla, California, 92038-2284, USA.) and according to the small scale rapid plasmid DNA isolation procedure described therein. The orientation of 5 of the resulting clones was checked using the restriction enzyme ClaI. A clone was chosen which gave the same ClaI pattern as fd-tet, but which had no BstE II sites.

In vitro mutagenesis of FDT5Bst was used to generate vectors that facilitated cloning of antibody fragments downstream of the gene III signal peptide and in frame with the gene III coding sequence. The oligonucleotide directed mutagenesis system, version 2 (Amersham International) was used with oligo 1 (figure 4) to create FDTPs/Bs. The sequence of FDTPs/Bs (figure 4) was confirmed using the sequenase version 2.0 kit (USB Corp., PO Box 22400, Cleveland, Ohio, 44122, USA.) with oligo 3 (figure 4) as a primer.

A second vector FDTPs/Xh (to facilitate cloning of single chain F_v fragments) was generated by mutagenising FDTPs/Bs with oligo 2 according to the method of Venkitaraman, A.R., Nucl. Acid Res. 17, p 3314. The

sequence of FDTPs/Xh (figure 4) was confirmed using the sequenase version 2.0 kit (USB Corp.) with oligo 3 as a primer.

Clearly, alternative constructions will be apparent to those skilled in the art. For example, M13 and/or its host bacteria could be modified such that its gene III could be disrupted without the onset of excessive cell death; the modified fd gene III, or other modified protein, could be incorporated into a plasmid containing a single stranded phage replication origin, such as pUC119, superinfection with modified phage such as K07 would then result in the encapsulation of the phage antibody genome in a coat partially derived from the helper phage and partly from the phage antibody gene III construct.

The detailed construction of a vector such as FDTPs/Bs is only one way of achieving the end of a phage antibody. For example, techniques such as sticky feet cloning/mutagenesis (Clackson, T. and Winter, G. 1989 Nucl. Acids. Res., 17, p 10163-10170) could be used to avoid use of restriction enzyme digests and/or ligation steps.

Example 2.

Insertion of Immunoglobulin F_V Domain into Phage Antibody

The plasmid scFv D1.3 myc (gift from G. Winter and A. Griffiths) contains V_H and V_L sequences from the antibody D1.3 fused via a peptide linker sequence to form a single chain F_V version of antibody D1.3. The sequence of the scF_V and surrounding sequences in scFvD1.3 myc is shown in figure 5.

The D1.3 antibody is directed against hen egg lysozyme (Harper, M. et al., 1987, Molec. Immunol. 24, 97-108) and the scFv form expressed in E. coli has the same specificity (A. Griffiths and G. Winter personal Communication).

Digestion of scFv D1.3 myc with PstI and XhoI, excises a fragment of 693 bp which encodes the bulk of the scFv. Ligation of this fragment into FDTPs/Xh cleaved with PstI

and XhoI gave rise to the construct FDTSCFVD1.3 encoding the gene III signal peptide and first amino acid fused to the complete D1.3 scFv, followed by the mature gene III protein from amino acid 2.

The vector FDTPs/Xh was prepared for ligation by digesting with the PstI and XhoI for 2 hours followed by digestion with calf intestinal alkaline phosphatase (Boehringer Mannheim UK Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG) at one unit/ul for 30 minutes at 37°C. Fresh calf intestinal alkaline phosphatase was added to a final total concentration of 2 units/ul and incubated for a further 30 minutes at 37°C. The reaction was extracted three times with phenol/chloroform, precipitated with ethanol and dissolved in water. The insert from scFvD1.3 myc was excised with the appropriate restriction enzymes, extracted twice with phenol/chloroform, precipitated with ethanol and dissolved in water. Ligations were carried out as described in example 1 except both vector and insert samples were at a final concentration of 5 ng/ul each. The formation of the correct construct was confirmed by sequencing as described in example 1.

To demonstrate that proteins of the expected size were produced, virions were concentrated by PEG precipitation as described above, and the equivalent of 2mls of supernatant was loaded onto an 18% SDS polyacrylamide gel. After electrophoresis, the gel was soaked in gel running buffer (50 mM Tris, 380 mM Glycine, 0.1% SDS) with 20% methanol for 15 minutes. Transfer to nitrocellulose filter was executed in fresh 1x running buffer/20% methanol using TE70 Semi Phor a semi-dry blotting apparatus (Hoeffer, 654 Minnesota Street, Box 77387, San Francisco, California 94107, USA.).

After transfer, the filter was blocked by incubation for 1 hour in a 2% solution of milk powder (Cadbury's Marvel) in phosphate buffered saline (PBS). Detection of F_y and V_H protein sequences in the phage antibody fusion proteins was effected by soaking the filter for 1 hour with a 1/1000 dilution (in 2% milk powder) of a rabbit polyclonal

antiserum raised against affinity purified, bacterially expressed F_V fragment (gift from G. Winter). After washing PBS (3 x 5 minute washes), bound primary antibody was detected using an anti-rabbit antibody conjugated to horseradish peroxidase (Sigma, Fancy Road, Poole, Dorset, BH17 7NH, UK.) for 1 hour. The filter was washed in PBS/0.1% triton X-100 and developed with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.02% cobalt chloride, 0.03% hydrogen peroxide in PBS.

The results show that with FDTVHD1.3 (from example 3) and FDTSCVFVD1.3, a protein of between 69,000 and 92,500 daltons is detected by the anti- F_V serum. This is the expected size for the fusion proteins constructed. This product is not observed in supernatants derived from fd-tet, FDT6Bst or FDTPs/Xh.

Example 3.

Insertion of Immunoglobulin V_H Domain into Phage Antibody

The V_H fragment from D1.3 was generated from the plasmid pSW1-VHD1.3-TAG1 (Ward, E.S. et al., 1989 supra.). Digestion with PstI and BstEII generates the fragment shown between positions 113 and 432 in figure 5. Cloning of this fragment into the PstI and BstEII sites of FDTPs/Bs gave rise to the construct FDTVHD1.3 which encodes a fusion protein with a complete V_H inserted between the first and third amino acids of the mature gene III protein (amino acid two has been deleted).

The methods used were exactly as in example 2 except that the vector used was FDTPs/Bs digested with PstI and BstEII.

Example 4.

Analysis of Binding Specificity of Phage Antibodies

The binding of the various phage antibodies to the specific antigen, lysozyme, was analysed using ELISA techniques. Phage antibodies were grown in E.coli and phage antibody particles were precipitated with PEG as in the materials and methods. Bound phage antibody particles were detected using polyclonal rabbit serum raised against the

closely related phage M13.

ELISA plates were prepared by coating 96 well plates (Falcon Microtest III flexible plate. Falcon: Becton Dickinson Labware, 1950 Williams Drive, Oxnard, California, 93030, USA.) with 200 μ l of a solution of lysozyme (1mg/ml unless otherwise stated) in 50 mM NaHCO_3 for 16-24 hours. Before use, this solution was removed, the plate rinsed several times in PBS and incubated with 200 μ l of 2% milk powder/PBS for 1 hour. After rinsing several times with PBS, 100 μ l of the test samples were added and incubated for 1 hour. Plates were washed (3 rinses in 0.05% Tween 20/PBS followed by 3 rinses in PBS alone). Bound phage antibodies were detected by adding 200 μ l/well of a 1/1000 dilution of sheep anti-M13 polyclonal antiserum (gift from G. Winter) in 2% milk powder/PBS and incubating for 1 hour. After washing as above, plates were incubated with biotinylated anti-sheep antibody (Amersham International) for 30 minutes. Plate were washed as above, and incubated with streptavidin-horseradish peroxidase complex (Amersham International). After a final wash as above, 0.5 mg/ml ABTS substrate in citrate buffer was added (ABTS=2'2'-azinobis(3-ethylbenzthiazoline sulphonate); citrate buffer =50 mM citric acid, 50 mM tri-sodium citrate at a ratio of 54:46). Hydrogen peroxide was added to a final concentration of 0.003% and the plates incubated for 1 hour. The optical density at 405 nm was read in a Titertek multiskan plate reader.

Figure 6 shows the effect of varying the amount of phage antibody. 100 μ l of various dilutions of PEG precipitated phage were applied and the amount expressed in terms of the original culture volume from which it was derived. Signals derived from both the scFv containing phage antibody (FDTSCFVD1.3) and the V_H containing phage antibody (FDTVHD1.3) were higher than that derived from the phage antibody vector (FDTPs/Xh). The highest signal to noise ratio occurs using the equivalent of 1.3 mls of culture.

Figure 7 shows the results of coating the plates with varying concentrations of lysozyme or bovine serum albumin (BSA). The equivalent of 1 ml of the original phage antibody culture supernatant was used. The signals from supernatants derived from FDTSCFVD1.3 were again higher than those derived from FDTPs/Xh when lysozyme coated wells were used. There was no significant difference between these two types of supernatant when the plates were coated with BSA. Broadly speaking the level of signal on the plates is proportional to the amount of lysozyme coated. These results demonstrate that the binding detected is specific for lysozyme as the antigen.

Example 5.

Construction of fd Cat 2

It would be useful to design vectors that enable the use of restriction enzymes that cut DNA infrequently, thus avoiding unwanted digestion of the antibody gene inserts within their coding sequence. Enzymes with an eight base recognition sequence are particularly useful in this respect, for example NotI and SfiI. Chaudhary et al (PNAS 87 p1066-1070, 1990) have identified a number of restriction sites which occur rarely in antibody variable genes. The applicant has designed and constructed a vector that utilises two of these sites, as an example of how this type of enzyme can be used. Essentially sites for the enzymes ApaI and NotI were engineered into FDTPs/Xh to create fdCAT2.

The oligonucleotide:

5'ACT TTC AAC AGT TTC TGC GGC CGC CCG TTT GAT CTC GAG CTC CTG CAG TTG GAC CTG TGC ACT GTG AGA ATA GAA 3'

was synthesised (supra fig 4 legend) and used to mutagenise FDTPs/Xh using an in vitro mutagenesis kit from Amersham International as described in example 1, to create fd-CAT2. The sequence of fd-CAT2 was checked around the site of manipulation by DNA sequencing. The final sequence around the insertion point within gene III is shown in figure 8.

Example 6

Specific Binding of Phage-antibody(pAb) to Antigen

The binding of pAb D1.3 (FDTSCFVD1.3 of example 2) to lysozyme was further analysed by ELISA.

Methods.

1. Phage growth.

Cultures of phage transduced bacteria were prepared in 10-100 mls 2 x YT medium with 15 µg/ml tetracycline and grown with shaking at 37°C for 16-24 hrs. Phage supernatant was prepared by centrifugation of the culture (10 min at 10,000 rpm, 8 x 50 ml rotor, Sorval RC-5B centrifuge). At this stage, the phage titre was $1 - 5 \times 10^{10}$ ml⁻¹ transducing units. The phage were precipitated by adding 1/5 volume 20% polyethylene glycol, 2.5 M NaCl, leaving for 1 hr at 4°C, and centrifuging (supra). The phage pellets were resuspended in 10 mM Tris-HCl, 1mM EDTA pH 8.0 to 1/100th of the original volume, and residual bacteria and aggregated phage removed by centrifugation for 2 min in a bench microcentrifuge.

ELISA

Plates were coated with antigen (1 mg ml⁻¹ antigen) and blocked as described in example 4. 2×10^{10} phage transducing units were added to the antigen coated plates in phosphate buffered saline (PBS) containing 2% skimmed milk powder (MPBS). Plates were washed between each step with three rinses of 0.5% Tween-20 in PBS followed by three rinses of PBS. Bound phage was developed by incubating with sheep anti-M13 antisera and detected with horseradish peroxidase (HRP) conjugated anti-goat serum (Sigma, Poole, Dorset, UK) and ABTS (2'2'-azinobis (3-ethylbenzthiazoline sulphonic acid)). Readings were taken at 405 nm after a suitable period. The results (figure 9) show that the antibody bearing-phage had the same pattern of reactivity as the original D1.3 antibody (Harper, M., Lema, F., Boulot, G., and Poljak, F.J. (1987) *Molec. Immunol.* 24, 97-108), and bound to hen egg-white lysozyme, but not to turkey egg-white lysozyme, human lysozyme or bovine serum albumin. The specificity of the phage is particularly illustrated by the

lack of binding to the turkey egg-white lysozyme that differs from hen egg-white lysozyme by only 7 amino acids.

Example 7

Expression of Fab D1.3 in fd CAT2

The aim of this example was to demonstrate that the scFv format used in example 2 was only one way of displaying antibody fragments in the pAb system. A more commonly used antibody fragment is the Fab fragment (figure 1) this example describes the construction of a pAb that expresses a Fab-like fragment on its surface and shows that it binds specifically to its antigen. The applicant chose to express the heavy chain of the antibody fragment consisting of the VH1 and CH1 domains in the pAb itself and to co-express the light chain in the same cell; the VH and CH1 regions of anti-lysozyme antibody D1.3 were cloned in fd CAT2, and the corresponding light chain cloned in plasmid pUC19. The light chain associates with the heavy chain-gene III fusion, as it does when the heavy and light chains are expressed as independent proteins (Skerra, A and Pluckthun, A. Science 240, p1038-1040 (1988)).

It is possible to express the light chain from within the pAb genome by, for example, cloning an expression cassette into a suitable place in the phage genome. Such a suitable place would be the intergenic region which houses the multicloning sites engineered into derivative of the related phage M13 (see, for example, Yanisch-Perron, C. et al., Gene 33, p103-119, (1985)).

The starting point for this example was the clone Fab D1.3 in pUC19, a map of which is shown in figure 10. The sequence encoding the VH-CH1 region was PCR amplified from Fab D1.3 in pUC19 using oligonucleotides KSJ 6 and 7, which retain the Pst I site at the 5' end and introduce a Xho I site at the 3' end, to facilitate cloning into fd CAT2. The sequences for the oligonucleotides KSJ 6 and 7 are shown below.

KSJ6: 5' AGG TGC AGC TGC AGG AGT CAG G 3'

KSJ7: 5' GGT GAC CTC GAG TGA AGA TTT GGG CTC AAC TTT C 3'

PCR conditions were as described in example II, except that thirty cycles of PCR amplification were performed with denaturation at 92°C for 45 seconds, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. The template used was TG1 cells containing Fab D1.3 in pUC19 resuspended in water and boiled (see example 12). This regime resulted in amplification of the expected fragment of approximately 600bp. This fragment was cut with Pst I and Xho I, purified from an agarose gel and ligated into Pst I/Xho I-cut fd CAT2 as described in example 12. Part of the ligation was transformed into E.coli MC1061 (Available from, for example, Clontech Laboratories Inc, Palo Alto, California) and colonies identified by hybridisation with the oligonucleotide D1.3CDR3A as described in example 10. The presence of the VHCH1 gene fragment was likewise confirmed by PCR, using oligonucleotides KSJ6 and 7. A representative clone was called fd CAT2: VHCH1 D1.3.

The heavy chain was deleted from fab D1.3 in pUC19 by Sph I cleavage of fab D1.3 plasmid DNA. The 2.7Kb fragment containing pUC19 and the light chain gene was purified from a TAE agarose gel, and 10ng of this DNA self-ligated and transformed into competent E.coli TG1. Cells were plated on 2YT agar containing ampicillin (100µg/ml) and incubated at 30°C overnight. The resulting colonies were used to make miniprep DNA (Sambrook et al. supra), and the absence of the heavy chain gene confirmed by digestion with Sph I and Hind III. A representative clone was called LC D1.3 DHC.

An overnight culture of fd CAT2: VHCH1 D1.3 cells was microcentrifuged at 13,000Xg for 10 minutes and 50µl of the supernatant containing phage particles added to 50µl of an overnight culture of LC D1.3 DHC cells. The cells were incubated at 37°C for 10 minutes and plated on 2YT agar containing ampicillin (100µg/ml) and 15mg/ml tetracycline. Phage were prepared from some of the resulting colonies and assayed for their ability to bind lysozyme as described in example 6.

The results (Figure 11) showed that when the heavy and

light chain Fab derivatives from the original antibody D1.3 were present, the pAb bound to lysozyme. pAb expressing the fd VHCH1 fragment did not bind to lysozyme unless grown in cells also expressing the light chain. This shows that a functional Fab fragment was produced by an association of the free light chain with the VHCH1 fragment fused to gene III and expressed on the surface of the pAb.

Example 8

Isolation of Specific, Desired Phage from a Mixture of Vector Phage.

The applicant purified pAb (D1.3) (originally called FDTSCFVD1.3 in example 2) from mixtures using antigen affinity columns. pAb (D1.3) was mixed with vector fd phage (see table 1) and approximately 10^{12} phage passed over a column of lysozyme-Sepharose (prepared from cyanogen bromide activated sepharose 4B (Pharmacia, Milton Keynes, Bucks, UK.) according to the manufacturers instructions. TG1 cells were infected with appropriate dilutions of the elutes and colonies derived, were analysed by probing with an oligonucleotide that detects only the pAb (D1.3) (see Table 1 and Fig. 12) A thousand fold enrichment of pAb (D1.3) was seen with a single column pass. By growing the enriched phage and passing it down the column again, enrichments of up to a million fold were seen.

Enrichment was also demonstrated using purely immunological criteria. For example, 10^{12} phage (at a ratio of 1 pAb (D1.3) to 4×10^6 FDTPs/Bs) was subjected to two rounds of affinity selection, and then 26 colonies picked and grown overnight. The phage was then assayed for lysozyme binding by ELISA (as example 6). Five colonies yielded phage with lysozyme binding activities, see table 1, and these were shown to encode the scFv (D1.3) by PCR screening (example 13, using 30 cycles of 1 minute at 92°C , 1 minute at 60°C , 1 minute at 72°C using CDR3PCR1 and oligo 3 (fig. 4) as primers).

Thus very rare pAbs can be fished out of large populations, by using antigen to select and then screen the phage.

Methods

Affinity Chromatography of pAbs

Approximately 10^{12} phage particles in 1ml MPBS were loaded onto a 1 ml lysozyme-Sepharose affinity column which had been prewashed in MPBS. The column was washed in turn with 10 ml PBS; then 10 ml 50 mM Tris-HCl, 500 mM NaCl pH 7.5; then 10ml 50 mM Tris-HCl, 500 mM NaCl pH 8.5; then 5 mls 50 mM Tris-HCl, 500 mM NaCl pH 9.5 (adjusted with triethylamine) and then eluted with 5 ml 100 mM triethylamine. The eluate was neutralised with 0.5 M sodium phosphate buffer pH 6.8 and the phage plated for analysis. For a second round of affinity chromatography, the first column eluate was plated to about 30,000 colonies per petri dish. After overnight growth, colonies were then scrape into 5 ml 2 x YT medium, and a 20 μ l aliquot diluted into 10 ml fresh medium and grown overnight. The phage was PEG precipitated as above, resuspended in 1 ml MPBS and loaded onto the column, washed and eluted as above.

Oligonucleotides synthesised:

CDR3PCR1 5'TGA GGA C(A or T) C(A or T)GC CGT CTA CTA CTG TGC
3'

Oligonucleotide probing

40 pmole oligonucleotide VH1FOR (Ward, E. S., et al (1989) Nature 341, 544-546), specific to pAb (D1.3) was phosphorylated with 100 μ Ci γ - 32 P ATP, hybridised (1pmole/ml) to nitrocellulose filters at 67°C in 6 x SSC buffer for 30 minutes and allowed to cool to room temperature for 30 mins, washed 3 x 1 min at 60°C in 0.1 x SSC.

Example 9

Construction of pAb Expressing Anti-hapten Activity

Oxazalone is a hapten that is a commonly used for studying

the detail of the immune response. The anti-oxazalone antibody, NQ11 has been described previously (E. Gherardi, R. Pannell, C. Milstein, J. Immunol. Method 126 61-68). A plasmid containing the VH and VL genes of NQ11 was converted to a ScFv form by inserting the BstEII/SacI fragment of SCFVD1.3 myc (nucleotides 432-499 of Fig 5) between the VH and VL genes to generate pSCFVNQ11, the sequence of which is shown in fig 13. This ScFv was cloned into the PstI/XhoI site of FdTPs/Xh (as described earlier) to generate pAb NQ11. (NQ11 has an internal PstI site and so it was necessary to do a complete digest of pSCFVNQ11 with XhoI followed by a partial digest with PstI)

The specific binding of pAb NQ11 was confirmed using ELISA. ELISA plates were coated at 37°C in 50mM NaHCO₃ at a protein concentration of 200 µg/ml. Plates were coated with either hen egg lysozyme (HEL), bovine serum albumin (BSA), or BSA conjugated to oxazalone (OX-BSA) (method of conjugation in Makela O., Kaartinen M., Pelkonen J.L.T., Karjalainen K. (1978) J.Exp.Med.148 1644). Preparation of phage, binding to ELISA plates, washing and detection was as described in example 6. Samples were assayed in duplicate and the average absorbance after 10 minutes presented in figure 14.

This result demonstrates that the pAb NQ11 binds the correct antigen. Figure 14 also shows that pAb D1.3 and pAb NQ11 bind only to the antigen against which the original antibodies were raised.

Example 10

Enrichment of pAb D1.3 from Mixtures of Other pAb by Affinity Purification

3×10^{10} phage in 10mls of PBSM at the ratios of pAb D1.3 to pAb NQ11 shown in table 2 were passed over a 1ml lysozyme Sepharose column. Washing, elution and other methods were as described in example 8 unless otherwise stated. Elutes from the columns were used to infect TG1 cells which were

then plated out. Colonies were probed with a probe which distinguishes pAb D1.3 from pAb NQ11. The sequence of this oligonucleotide (D1.3CDR3A) is:-

5'GTA GTC AAG CCT ATA ATC TCT CTC 3'

Table 2 presents the data from this experiment. An enrichment of almost 1000 fold was achieved in one round and an enrichment of over a million fold in two rounds of purification. This parallels the result described in example 8.

Example 11

Insertion of the Extracellular Domain of the Human Receptor for Platelet Derived Growth Factor Isoform BB into fdCAT-2

A gene fragment encoding the extracellular domain of the human receptor for platelet derived growth factor isoform BB (h-PDGFB-R) was isolated by amplification using the polymerase chain reaction, of plasmid RP41 (from the American Type Culture collection, Cat. No. 50735), a cDNA clone encoding amino acids 43 to 925 of the PDGF-B receptor (Gronwald, R.G.K. et al., PNAS 85, p3435-3439 (1988), amino acids 1 to 32 constitute the signal peptide). The oligonucleotide primers were designed to amplify the region of the h-PDGFB-R gene corresponding to amino acids 43 to 531 of the encoded protein. The primers also incorporate a unique ApaI site at the 5' end of the fragment and a unique XhoI site at the 3' end, to facilitate cloning into the vector fdCAT-2. The sequence of the primers is:

RPDGF1 5'C ACA GTG CAC GTC CTC AAT GTC TCC AGC ACC TTC 3'

RPDGF2 5'GAT CTC GAG CTT AAA GGG CAA GGA GTG TGG CAC 3'

PCR amplification was performed using high fidelity conditions (Eckert, K.A. and Kunkel, T.A., 1990 Nucl. Acids. Research 18 p3739-3744). The PCR mixture contained; 20mM Tris HCl (pH7.3 at 70°C), 50mM KCl, 4mM magnesium chloride, 0.01% gelatin, 1mM each of dATP, dGTP, dCTP, and dTTP, 500ng/ml RP41 DNA, 1µM each primer and 50 units/ml Taq polymerase (Cetus/Perkin-Elmer, Beaconsfield, Bucks, UK)

Thirty cycles of PCR were performed with denaturation at 92°C for 1 minute, annealing at 50°C for 2 min and extension at 72°C for 3 min. This reaction resulted in amplification of a fragment of ca 1500bp as expected.

fdCAT-2 vector DNA was digested with ApaI and XhoI (New England Biolabs) according to manufacturers recommendations, extracted with phenol/chloroform and ethanol precipitated (Sambrook et al., supra). Cloning of amplified RP41 DNA into this vector and identification of the desired clones was performed essentially as in example 12 except that probing was with 32-P labelled RPDGF1 and analytical PCR was performed using RPDGF1 and RPDGF2 as primers.

Example 12

Insertion of the Extracellular Domain of the Human Epidermal Growth Factor Receptor into fd-CAT2

A gene fragment encoding the extracellular domain of the human epidermal growth factor receptor (hEGF-R) was isolated by polymerase chain amplification of plasmid pJ3EGF-R (Clark et al. (1988) J. Cell Physiol. 134, p421-428) which contains the gene for hEGF-R (Ullrich, A. et al., Nature 309, p418-425, (1984)). The oligonucleotide primers used were designated to amplify the region of the hEGF-R gene corresponding to amino acid 1 of the mature protein through to amino acid 621. The primers also incorporate an unique ApaI site at the 5' end of the fragment and an unique Xho I site at the 3' end, to facilitate cloning into the vector fd-CAT2. The sequence of the primers is:

Oligo KSJ4: 5' GAT CTC GAG GGA CGG GAT CTT AAG CCC ATT CGT TGG 3'

Oligo KSJ5: 5' CAG AGT GCA CTG GAG GAA AAG AAA GTT TGC CA 3'

PCR amplification was carried out using high-fidelity conditions (Eckert, K.A. and Kunkel, T.A. 1990 Nucl. Acids Res. 18, 3739-3744). The PCR mixture contained: 20mM TrisHCl (pH 7.3 at 70°C), 50mM KCl, 4mM MgCl₂, 0.01%

gelatin, 1mM each of dATP, dGTP, dCTP and dTTP, 500ng/ml pJ3 plasmid, 0.5 μ M each primer and 50U/ml Taq polymerase (Cetus/Perkin-Elmer). Thirty cycles of PCR amplification were performed with denaturation at 92°C for 1 minute, annealing at 50°C for 2 minutes and extension at 72°C for 3 minutes. This regime resulted in amplification of a fragment of the expected size (approximately 1800bp).

The PCR mixture was extracted with phenol/chloroform and ethanol precipitated (Sambrook et al. supra.) before digestion with ApaI 1 and Xho 1 (New England BioLabs) according to manufacturers recommendations. The fragment was resolved on a 1% Tris-Acetate-EDTA agarose gel (Sambrook et al. supra.) and purified using Geneclean (BIO 101) (Geneclean, La Jolla, San Diego, California, USA) according to manufacturers recommendations.

fd-CAT2 vector DNA was digested with ApaI 1 and Xho 1 (New England BioLabs) according to manufacturers recommendations, extracted with phenol/chloroform and ethanol precipitated (Sambrook et al. supra.).

75ng of ApaI 1/Xho 1-digested vector DNA was ligated to 40ng of PCR-amplified ApaI 1/Xho I-digested hEGF-R fragment in 12 μ l of ligation buffer (66mM TrisHCl (pH7.6), 5mM MgCl₂, 5mM dithiothreitol, 100mg/ml bovine serum albumin, 0.5mM ATP, 0.5mM Spermidine) and 400 units T4 DNA ligase (New England BioLabs) for 16 hours at 16°C.

Two μ l of the ligation mixture was transformed into 200 μ l of competent E.coli MC1061 cells, plated on 2YT agar containing 15mg/ml tetracycline and incubated at 30°C for 20 hours.

Colonies containing hEGF-R were identified by probing with 32p-labelled KSJ 4 oligonucleotide (Sambrook et al. supra.) and the presence of an insert in hybridising colonies confirmed by PCR using the conditions described above. In this case the template DNA was prepared from the colonies by picking some colony material into 100 μ l of

distilled water and boiling for 10 minutes. 1µl of this mixture was used in a 20µl PCR.

Example 13.

Insertion of a Gene Encoding an Enzyme (Alkaline phosphatase) into fd-CAT2

As an example of the expression of a functional enzyme on the bacteriophage surface, the applicants have chosen bacterial alkaline phosphatase an enzyme that normally functions as a dimer (McCracken, S. and Meighen, E., J. Biol. Chem. 255, p2396-2404, (1980)). The oligonucleotides were designed to generate a PCR product with an Apa Ll site at the 5' end of phoA gene and a Not I site at its 3' end, thus facilitating cloning into fd-CAT 2 to create a gene III fusion protein. The oligonucleotides synthesised were:

phoA1:5' TAT TCT CAC AGT GCA CAA ACT GTT GAA CGG ACA CCA GAA ATG CCT GTT CTG 3' and,

phoA2:5' ACA TGT ACA TGC GGC CGC TTT CAG CCC CAG AGC GGC TTT C3'

The sequence of the phoA gene is presented in Chang C. N. et al., Gene 44, p121-125 (1986).

The PCR reaction was carried out in 100µl containing 50mM KCl, 2.5mM MgCl₂, 0.01% gelatin, 10mM Tris/HCl pH 8.3, 0.25 units/µl of Taq polymerase (Cetus/Perkin Elmer) and 0.5µg/ml template. The template was the pEK plasmid (described by Chaidaroglou et al., Biochemistry 27 p8338-8343, 1988). The PCR was carried out in a Techne (Techne, Duxford, Cambridge, UK) PHC-2 dri-block using thirty cycles of 1 min at 92°C, 2 min at 50°C, 3 min at 72°C.

The resultant product was extracted with phenol:chloroform, precipitated with ethanol, and the pellet dissolved in 35µl water. Digestion with 0.3 units/µl of Apl Ll was carried out in 150µl volume according to manufacturers instructions for two hours at 37°C. After heat inactivation of the enzyme at 65°C, NaCl was added to a final concentration of 150mM and 0.4 units/µl NotI enzyme.

added. After incubation for 2 hours at 37°C, the digest was extracted with phenol:chloroform and precipitated as above, before being dissolved in 30µl of water. The vector fd-CAT2 was sequentially digested with Apa I and NotI according to the manufacturers instructions and treated with calf intestinal alkaline phosphatase as described in example 2. The sample was extracted three times with phenol:chloroform, precipitated with ethanol and dissolved in water. The ligations were performed with a final DNA concentration of 1-2ng/µl of both the cut fd-CAT2 and then digested PCR product. The ligations were transformed into competent TG1 cells and plated on 2xTY tet plates. Identification of clones containing the desired insert was by analytical PCR performed using the conditions and primers above on boiled samples of the resulting colonies. The correct clone containing the phoA gene fused in frame to gene III was called fd-phoA1. The sequence at the junction of the cloning region is given in figure 15.

Example 14

Measuring Enzyme Activity of Phage-enzyme

Overnight cultures of TG1 or KS272 (E.coli cells lacking phoA. Strauch K. L., and Beckwith J. PNAS 85 1576-1580, 1988) cells containing either fd-phoA1 or fd-CAT2 were grown at 37°C in 2xTY with 15µg/ml tetracycline. Concentrated PEG precipitated phage were prepared as described earlier. Enzyme assays (Malamy, M.H. and Horecker B.L., Biochemistry 3, p1893-1897, (1964)) were carried out at 24°C in a final concentration of 1M Tris/HCl pH 8.0, 1mM nitrophenyl phosphate (Sigma), 1mM MgCl₂. 100µl of a two times concentrate of this reaction mixture was mixed with 100µl of the test sample in a 96 well plate. Absorbance readings were taken every minute for 30 minutes at a wavelength of 405nm in a Titretek Mk 2 plate reader. Initial reaction rates were calculated from the rate of change of absorbance using a molar absorbance of 17000.

Standard curves (amount of enzyme vs. rate of change of absorbance) were prepared using dilutions of purified bacterial alkaline phosphatase (Sigma type III) in 10mM Tris/HCl pH 8.0, 1mM EDTA. The number of enzyme molecules in the phage samples were estimated from the actual rates of change of absorbance of the phage samples and comparison to this standard curve.

The results in Table 3 show that alkaline phosphatase activity was detected in PEG precipitated material in the sample containing fd-phoA1 but not fd-CAT2. Furthermore, the level of activity was consistent with the expected number of 1-2 dimer molecules of enzyme per phage. The level of enzyme activity detected was not dependant on the host used for growth. In particular, fd-phoA1 grown on phoA minus hosts showed alkaline phosphatase activity.

Therefore, the phage express active alkaline phosphatase enzyme from the phoA-gene III fusion on the phage surface.

Example 15

Insertion of Binding Molecules into Alternative Sites in the Phage

The availability of an alternative site in the phage for the insertion of binding molecules would open up the possibility of expressing more than one antibody fragment in a single pAb. This may be used to generate single or multiple binding specificities. The presence of two distinct binding activities on a single molecule will greatly increase the utility and specificity of this molecule. It may be useful in the binding of viruses with a high mutational rate such as human immunodeficiency virus. In addition, it may be used to bring antigens into close proximity (eg. drug targetting or cell fusion) or it may act as a "molecular clamp" in chemical, immunological or enzymatic processes.

The vector fd-tet and the derivatives described here, have a single BamHI site in gene 3. This has previously

been used for the expression of peptide fragments on the surface of filamentous bacteriophage (Smith GP. (1985) Science 228 p1315-1317 and de la Cruz et al. (1988) J Biol. Chem. 263 p4318-4322). This provides a potential alternative site for the insertion of antibody fragments.

DNA fragments encoding SCFv's from D1.3 or NQ11 were generated by PCR using the primers shown below. These primers were designed to generate a fragment with BamH1 sites near both the termini, to enable cloning into the BamH1 site of gene3 (see figure 16(1)). The oligonucleotides used, also ensure that the resulting PCR product lacks Pst1 and Xho1 restriction sites normally used for manipulating the SCFv's (see figure 16(1)). This will facilitate subsequent manipulation of a second antibody fragment in the usual way at the N terminus of gene 3. The oligonucleotides used were:-

G3Bam1 5'TTT AAT GAG GAT CCA CAG GTG CAG CTG CAA GAG 3'

G3Bam2 5' AAC GAA TGG ATC CCG TTT GAT CTC AAG CTT 3'.

Preparation of vector and PCR insert

The PCR reaction was carried out in an 80 µl reaction as described in example 13 using 1ng/µl of template and 0.25U/µl of Taq polymerase and a cycle regime of 94°C for 1 minute, 60°C for 1 minute and 70°C for 2 minutes over 30 cycles. The template was either pSCFvNQ11 (example 9) or SCFvD1.3 myc (example 2). Reaction products were extracted with phenol:chloroform, precipitated, dissolved in water and digested with BamH1 according to manufacturers instructions. The digest was re-extracted with phenol:chloroform, precipitated and dissolved in water.

The vector FDTPs/Xh was cleaved with BamH1 and treated with calf intestinal phosphatase and purified as described in example 2. Ligations were set up at a vector concentration of approximately 6ng/µl and a PCR insert concentration of approximately 3ng/µl. These were ligated for 2.5 hours at room temperature before transforming into

competent TGI cells and plating on TYE tet plates. The resultant colonies were probed as described in example 8. DNA was prepared from a number of colonies and the correct orientation and insert size confirmed by restriction digestion with Hind III in isolation or in combination with BamHI. (One Hind III site is contributed by one of the primers and the other by the vector).

Two clones containing a D1.3 insert (FDTBam1 and FDTBam2) and one containing an NQ11 insert (NQ11Bam1) were grown up and phage prepared as described earlier. ELISAs were carried out as described in example 6. No specific signal was found for any of these clones suggesting that the natural BamHI site is not a suitable site for insertion of a functional antibody (results not shown).

It may be possible to clone into alternative sites to retain binding activity. The peptide repeats present in gene3 may provide such a site (figure 16 blocks A and B). This can be done by inserting a BamHI site and using the PCR product described above. To facilitate this, the natural BamHI site was removed by mutagenesis with the oligonucleotide shown below (using an in vitro mutagenesis kit (Amersham International)):-

G3mut6Bam 5' CA AAC GAA TGG GTC CTC CTC ATT A 3'

The underlined residue replaces an A residue thereby removing the BamHI site. DNA was prepared from a number of clones and several mutants lacking BamHI sites identified by restriction digestion.

The oligonucleotide G3 Bamlink was designed to introduce a BamHI site at a number of possible sites within the peptide linker sites A and B, see figure 16(2). The sequence of the linker is:

5'CC (G or A) CC ACC CTC GGA TCC (G or A) CC ACC CTC 3'

Its relationship to the peptide repeats in gene III is shown in figure 16.

Example 16PCR Assembly of Mouse VH and VL kappa (VLK) Repertoires
for Phage Display

5

The principle is illustrated in figure 17. Details are provided in sections A to F below but the broad outline is first discussed.

1. cDNA is prepared from spleen RNA from an appropriate
 10 mouse and the VH and VLK repertoires individually amplified. Separately, primers ~~reverse and complementary to VH1FOR 2 (domain 1) and VLK2BACK/~~
~~(domain 2)~~ are used to amplify an existing scFv-containing DNA by PCR. (The term FOR refers to eg.
 15 a primer for amplification of sequences on the sense strand resulting in antisense coding sequences. The term BACK refers to eg. a primer for amplification of sequences on the antisense strand resulting in sense coding sequences). This generates a 'linker'
 20 molecule encoding the linker with the amino acid sequence (1 letter code) (GGGGS)₃ which overlaps the two primary (VH and VLK) PCR products.
2. The separate amplified VH, VLK and linker sequences
 now have to be assembled into a continuous DNA
 25 molecule by use of an 'assembly' PCR. In the secondary 'assembly' PCR, the VH, VLK and linker

bands are combined and assembled by virtue of the above referred to overlaps, to generate an assembled PCR band with VHs and VLKs randomly spliced in frame for expression as scFVs.

5 The assembly PCR is carried out in two stages. Firstly, 7 rounds of cycling with just the three bands present in the PCR, followed by a further 20 rounds in the presence of the flanking primers VH1BACK ~~(referring to domain 1 of VH)~~ and VLKFOR.

10 The nucleotide sequences for these oligonucleotide primers are provided under the section entitled 'Primer Sequences' below. This two stage process, avoids the potential problem of preferential amplification of the first combinations to be

15 assembled.

3. For cloning into the phage system, the assembled repertoires must be 'tagged' with the appropriate restriction sites. In the example provided below this is illustrated by providing an ApaI1 restriction site at the VH end of the continuous DNA

20 molecule and a Not 1 site at the VLK end of the molecule. This is carried out by a third stage PCR using tagged primers. The nucleotide sequences for these oligonucleotide primers are also provided

25 under the section entitled 'Primer Sequences' below. There are however, 4 possible kappa light chain

J sequences. ~~(whereas there is only a single heavy chain sequence)~~ Therefore 4 oligonucleotide primer sequences are provided for VLK.

5 For this third stage PCR, sets of primers which have 4 and 10 nucleotides after the restriction sites have been used. However, long tags may give better cutting, in which case 15-20 nucleotide overhangs could be used.

10 Scrupulously clean procedures must be used at all times to avoid contamination during PCR. Negative controls containing no DNA must always be included to monitor for contamination. Gel boxes must be depurinated. A dedicated Geneclean kit (B10 101, Geneclean, La Jolla, San Diego, California, USA) can be
15 used according to manufacturers instructions to extract DNA from an agarose gel. The beads, NaI and the NEW wash should be aliquoted.

All enzymes were obtained from CP Laboratories, P.O. Box 22, Bishop's Stortford, Herts CM20 3DH and the
20 manufacturers recommended and supplied buffers were used unless otherwise stated.

A. RNA Preparation

RNA can be prepared using many procedures well known
25 to those skilled in the art. As an example, the following protocol (Triton X-100 lysis, phenol/SDS RNase

- inactivation) gives excellent results with spleen and hybridoma cells (the addition of ^{RVC} ~~WVC~~ (veronal ribocycl complex) as an RNase inhibitor is necessary for spleen cells). Guanidinium isothiocyanate/CsCl procedures
- 5 (yielding total cellular RNA) also give good results but are more time-consuming.
1. Wash $1 - 5 \times 10^7$ freshly harvested cells in 50 ml PBS at 800g for 10 minutes.
 2. On ice, add 1 ml ice-cold lysis buffer (see later
10 for details) to the pellet and resuspend it with a 1 ml Gilson pipette. Leave on ice for 5 minutes.
 3. Spin for 5 minutes at 4°C in a microfuge at 13000 rpm, in precooled tubes.
 4. Transfer 0.5 ml of the supernatant to each of two
15 eppendorf tubes containing 60 μl 10% (w/v) SDS and 250 μl phenol (equilibrated with 100 mM Tris-HCl pH 8.0). Vortex hard for 2 minutes, then microfuge (13000rpm) for five minutes at room temperature.
 5. Re-extract the aqueous upper phase five times with
20 0.5 ml of phenol.
 6. Precipitate with 1/10 volume 3M sodium acetate and 2.5 volumes ethanol at 20°C overnight or dry ice-isopropanol for 30 minutes.
 7. Wash the RNA pellet and resuspend in 50 μl water.
 - 25 Use 2.5 ^{μl} to check concentration by measuring the optical density at 260nm and check 2 μg on a 1% agarose gel. 40 μg

, New England Biologicals Ltd. as supplied by
C.P. Laboratories Ltd., address given above,
catalogue no. 1402

to 107µg of RNA was obtained from spleen cells derived from mice.

Lysis buffer is [10mM Tris-HCl pH 7.4, 1mM MgCl₂,
 5 150mM NaCl, 10mM ^{RVC}~~ATG~~ (New England Biolabs), 0.5% (w/v)
 Triton X-100]; prepared fresh.

B. cDNA Preparation

cDNA can be prepared using many procedures well know to
 10 those skilled in the art. As an example, the following
 protocol can be used:

1. Set up the following reverse transcription mix:

	<u>µl</u>
H ₂ O (DEPC-treated)	20
15 5mM dNTP	10
10 x first strand buffer	10
0.1M DTT	10
FOR primer(s) (10 pmol/µl)	2 (each) (see below)
RNasin (Promega; 40 U/µl)	4

20

NB

- i) DEPC is diethylpyrocarbonate, the function of which
 is to inactivate any enzymes that could degrade DNA
 or RNA
- 25 ii) dNTP is deoxynucleotide triphosphate
- iii) DTT is dithiothreitol the function of which is as an

antioxidant to create the anaerobic environment necessary for enzyme function.

iv) RNasin is a ribonuclease inhibitor obtained from Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA.

2. Dilute 10 µg RNA to 40 µl final volume with DEPC-treated water. Heat at 65°C for 3 minutes and hold on ice for one minute (to remove secondary structure).
3. Add to the RNA the reverse transcription mix (58 µl) and 4 µl of the cloned reverse transcriptase 'Super RT' (Anglian Biotech Ltd., Whitehall House, Whitehall Road, Colchester, Essex) and incubate at 42°C for one hour.
4. Boil the reaction mix for three minutes, cool on ice for one minute and then spin in a microfuge to pellet debris. Transfer the supernatant to a new tube.

20

10 x first strand buffer is [1.4M KCl, 0.5M Tris-HCl pH 8.1 at 42°C 80mM MgCl₂].

of the RNA

The primers anneal to the 3' end. Examples of kappa light chain primers are MJK1FONX, MJK2FONX, MJK4FONX and MJK5FONX (provided under 'Primer Sequences' below) and examples of heavy chain primers are CTG GAC AGG GAT CCA

GAG TTC CA and CTG GAC AGG GCT CCA TAG TTC CA. The two heavy chain primers are provided as alternatives, the four light chain primers are provided for kappa light chains 1-4. Primers annealing to CH1, VLK and VL domains could also be used.

C. Primary PCRs

For each PCR and negative control, the following reactions are set up. In the following, the Vent DNA polymerase sold by C.P. Laboratories Ltd. (New England Biolabs) address given above. The buffers are as provided by C.P. Laboratories.

	<u>μl</u>
H ₂ O	32.5
10 x Vent buffer	5
20 x Vent BSA	2.5
5mM dNTPs	1.5
FOR primer 10 pMol/ μ l)	2.5
BACK primer 10pmol/ μ l)	2.5

20

The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. For VH, the FOR primer is VH1FOR-2 and the BACK primer is VH1BACK. For VLK the FOR primers are MJK1FONX, MJK2FONX, MJK4FONX and MJK5FONX (for the four respective kappa light chains) and the BACK primer is VK2BACK. Only one kappa light chain BACK

primer is necessary, because binding is to a nucleotide sequence common to the four kappa light chains.

UV this mix 5 minutes. Add 2.5 μ l cDNA preparation (from B above), 2 drops paraffin oil (Sigma Chemicals, Poole, Dorset, UK). Place on a cycling heating block, e.g. PHC-2 manufactured by Techne Ltd. Duxford UK, pre-set at 94°C. Add 1 μ l Vent DNA polymerase under the paraffin. Amplify using 25 cycles of 94°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

10 Purify on a 2% 1mp (low melting point agarose/TAE (tri-acetate EDTA) gel and extract the DNA to 20 μ l H₂O per original PCR using a Geneclean kit (see earlier) in accordance with the manufacturers instructions.

15 D. Preparation of linker

Set up in bulk (eg. 10 times):

	<u>μl</u>
H ₂ O	34.3
20 10 x Vent Buffer	5
20 x Vent BSA	2.5
5mM dNTPs	2
LINKFOR primer (10 pmol/ μ l)	2.5
LINKBACK primer (10 pmol/ μ l)	2.5
25 DNA from Exon 2 ^{D1.3} (example 2)	1
Vent enzyme	0.2

FDTSCVFVD1.3

The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. The FOR primer is LINKFOR and the BACK primer is LINKBACK.

- 5 Cover with paraffin and place on the cycling heating block (see above) at 94°C. Amplify using 25 cycles of 94°C 1 min, 65°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

- 10 Purify on 2% lmp/TAE gel (using a loading dye without bromophenol blue as a 93bp fragment is desired) and elute with SPIN-X column (Costar Limited, 205 Broadway, Cambridge, Ma. USA.,) and precipitation. Take up in 5µl H₂O per PCR reaction.

15 E. Assembly PCRs

A quarter of each PCR reaction product (5µl) is used for each assembly. The total volume is 25µl.

For each of the four VLK primers, the following are set up:

20

H ₂ O	4.95
10 x Vent buffer	2.5
20 X Vent BSA	1.25
5mM dNTPs	0.8

- 25 UV irradiate this mix for 5 min. Add 5µl each of Vh and Vk band and 1.5µl of linker as isolated from the

preparative gels and extracted using the Geneclean kit as described in C and D above. Cover with paraffin. Place on the cycling heating block preset at 94°C. Add 1µl Vent under the paraffin. Amplify using 7 cycles of 94°C
 5 2 min, 72°C 4 min. Then return the temperature to 94°C.

Add 1.5µl each of VH1BACK and the appropriate VKFOR primers MJK1FONX, MJK2FONX, MJK4FONX or MJK5FONX (10 pmol/µl) at 94°C. The primers should have been UV-
 10 treated as above. Amplify using 20 cycles of 94°C 1.5 min, 72°C 2.5 min. Post-treat at 60°C for 5 min. Purify on 2% lmp/TAE gel and extract the DNA to 20µl H₂O per assembly PCR using a Geneclean kit (see earlier) in accordance with the manufacturers instructions.

15

F. Adding Restriction Sites

For each assembly and control set up:

		<u>µl</u>
20	H ₂ O	36.5
	10 x Taq buffer	5
	5mM dNTPs	2
	FOR primer (10 pmol/µl)	2.5
	BACK primer (10 pmol/µl)	2.5
25	Assembly product	1

The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. The FOR primer is any of JK1NOT10, JK2NOT10, JK4NOT10 or JK5NOT10 (for the four respective kappa light chains) for putting a NotI
 5 restriction site at the VLK end. The BACK primer is HBKAPA10 for putting an ApaI restriction site at the VH end.

Cover with paraffin and place on the cycling heating block preset at 94°C. Add 0.5 µl Cetus Taq DNA
 10 polymerase (Cetus/perkin-Elmer, Beaconsfield, Bucks, UK) under the paraffin. Amplification is carried out using 11 to 15 rounds of cycling (depends on efficiency) at 94°C 1 min, 55°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

15 10 x Taq buffer is [0.1M Tris-HCl pH 8.3 at 25°C, 0.5M KCl, 15mM MgCl₂, 1mg/ml gelatine].

G. Work-up

Purify once with CHCl₃/IAA (isoamylalcohol), once
 20 with phenol, once with CHCl₃/IAA and back-extract everything to ensure minimal losses. Precipitate and wash twice in 70% EtOH. Dissolve in 70µl H₂O.

Digest overnight at 37°C with NotI:	<u>µl</u>
25 DNA (joined seq)	70
NEB NotI buffer x 10	10

NEB BSA x 10 10

Not1 (10 U/ μ l) 10

The DNA (joined sequence) above refers to the assembled DNA sequence comprising in the 5' to 3' direction

5 ApaL1 restriction site

VH sequence

Linker sequence

VLK sequence

Not 1 restriction site.

10 The VLK sequence may ^{contain} ~~be~~ any one of four possible
 J kappa/chain sequences.

15 The enzymes Not 1 above, ApaL1 below and the buffers NEB Not 1, NEB BSA above and the NEB buffer 4 (below) are obtainable from CP Laboratories, New England Biolabs mentioned above.

Re-precipitate, take up in 80 μ l H₂O. Add to this 10 μ l NEB buffer 4 and 10 μ l ApaL1.

Add the enzyme ApaL1 in aliquots throughout the day, as it has a short half-life at 37°C.

20 Purify on 2% 1mp/TAE gel and extract the DNA using a Geneclean kit, in accordance with the manufacturers instructions. Redigest if desired.

F. Final DNA product

25 The final DNA product is an approximate 700 bp fragment with Apa L1 and Not1 compatible ends consisting

of randomly associated heavy and light chain sequences linked by a linker. A typical molecule of this type is the scFvD1.3 molecule incorporated into fdscFvD1.3 described in example 3. These molecules can then be
 5 ligated into suitable fd derived vectors, e.g. fdCAT2 ~~by~~
~~fd CAT2 (example 1)~~, using standard techniques.

Primer sequences.

W = A or T R = A or G S = G or C M = A or C

10 Primary PCR oligos (restriction sites underlined):

VH1FOR-2 TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC

VH1BACK AGG TSM ARC TGC AGS AGT CWG G

MJK1FONX CCG TTT GAT TTC CAG CTT GGT GCC

MJK2FONX CCG TTT TAT TTC CAG CTT GGT CCC

15 MJK4FONX CCG TTT TAT TTC CAA CTT TGT CCC

MJK5FONX CCG TTT CAG CTC CAG CTT GGT CCC

VK2BACK GAC ATT GAG CTC ACC CAG TCT CCA

PCR oligos to make linker:

20 LINKFOR TGG AGA CTC GGT GAG CTC AAT GTC

LINKBACK GGG ACC ACG GTC ACC GTC TCC TCA

For adding restriction sites:

HBKAPA10 CAT GAC CAC AGT GCA CAG GTS MAR CTG CAG SAG TCW

25 GG

JK1NOT10 GAG TCA TTC TGC GGC CGC CCG TTT GAT TTC CAG CTT

GGT GCC

JK2NOT10 GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAG CTT

GGT CCC

JK4NOT10 GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAA CTT

5 TGT CCC

JK5NOT10 GAG TCA TTC TGC GGC CGC CCG TTT CAG CTC CAG CTT

GGT CCC

Example 17

10 Insertion of the Extra~~z~~cellular Domain of the Human
Receptor for Platelet Derived Growth Factor PDGF isoform
BB into fdCAT2

This example is substantially equivalent to example 11. However, in example 11, amino acids 33-42 of the
 15 mature protein were not provided. In this example, the primer RPDGF3 also includes bases encoding these amino acids.

A gene fragment encoding the extracellular domain of the human receptor for platelet derived growth factor
 20 isoform BB (h-PDGFB-R) was isolated by amplification, using the polymerase chain reaction, of plasmid RP41 (from the American Type Culture collection, Cat. No. 50735), a cDNA clone encoding amino-acids 43 to 925 of the PDGF-B receptor (Gronwald, R.G.K. et al PNAS 85
 25 p3435-3439 (1988), amino acids 1 to 32 constitute the signal peptide). The oligonucleotide primers were

designed to amplify the region of the h-PDGFB-R gene corresponding to amino acids 43 to 531 of the encoded protein. The primer RPDGF3 for the N-terminal region also included bases encoding amino acids 33 to 42 of the h-PDGFB-R protein (corresponding to the first ten amino acids from the N-terminus of the mature protein) to enable expression of the complete extracellular domain. The primers also incorporate a unique ApaI site at the N-terminal end of the fragment and a unique XhoI site at the C terminal end to facilitate cloning into the vector fdCAT2. The sequence of the primers is:

RPDGF3 5' CAC AGT GCA CTG GTC GTC ACA CCC CCG GGG CCA GAG
CTT GTC CTC AAT GTC TCC AGC ACC TTC GTT CTG 3'

RPDGF2 5' GAT CTC GAG CTT AAA GGG CAA GGA GTG TGG CAC 3'

PCR amplification was performed using high fidelity conditions (Eckert, K.A. and Kunkel, T.A. 1990 Nucl Acids Research 18 3739-3744). The PCR mixture contained: 20mM TrisHCl (pH7.3 at 70°C, 50mM KCl, 4mM magnesium chloride, 0.01% gelatin, 1mM each of dATP, dCTP, dGTP and dTTP, 500ng/ml RP41 DNA, 1µM each primer and 50units/ml Taq polymerase (Cetus/Perkin Elmer, Beaconsfield, Bucks, U.K.). Thirty cycles of PCR were performed with denaturation at 92°C for 1 min, annealing at 60°C for 1

min and extension at 72°C for 1.5 min. This reaction resulted in amplification of a fragment of ca. 1500bp as expected.

fdCAT2 vector DNA (see example 5) was digested with
5 ApaL1 and Xho1 (New England Biolabs) according to
manufacturers recommendations, extracted with
phenol/chloroform and ethanol precipitated (Sambrook et
al, supra). Cloning of amplified RP41 DNA into this
vector and identification of the desired clones was
10 performed essentially as in example 12 except that
probing was with 32-P labelled RPDGF2 and analytical PCR
was performed using RPDGF3 and RPDGF2.

Example 18

Binding of 125 I-PDGF-BB to the Extracellular Domain of the Human Receptor for Platelet Derived Growth Factor Isoform BB Displayed on the Surface of fd Phage.

5 Measured using an Immunoprecipitation Assay.

Phage particles, expressing the extracellular domain
of the human platelet derived growth factor isoform BB
receptor (fd h-PDGFB-R), were prepared by growing E.coli
MC1061 cells transformed with fd h-PDGFB-R in 50ml of
10 2xTY medium with 15ug/ml tetracyclin for 16 to 20 hours.
Phage particles were concentrated using polyethylene
glycol as described in example 6 and resuspended in PDGF
binding buffer (25mM HEPES, pH7.4, 0.15mM NaCl, 1mM

magnesium chloride, 0.25% BSA) to 1/33rd of the original volume. Residual bacteria and undissolved material were removed by spinning for 2 min in a microcentrifuge. Immunoblots using an antiserum raised against gene III
5 (Prof. I. Rashed, Konstanz., Germany) show the presence in such phage preparations of a geneIII-h-PDGFB-R protein of molecular mass 125000 corresponding to a fusion between h-PDGFB-R external domain (55000 daltons) and geneIII (apparent molecular mass 70000 on SDS-
10 polyacrylamide gel).

Duplicate samples of 350µl concentrated phage were incubated with ^{125}I -PDGF-BB (78.7fmol, 70nCi, 882Ci/mmol; Amersham International plc, Amersham, Bucks) for 1 hour at 37°C. Controls were included in which fdTPs/Bs vector
15 phage (figure 4(26)) or no phage replaced fd h-PDGFB-R phage. After this incubation, 10µl of sheep anti-M13 polyclonal antiscrum (a gift from M. Hobart) was added and incubation continued for 30 min at 20°C. To each sample, 40µl (20µl packed volume) of protein G Sepharose
20 Fast Flow (Pharmacia, Milton Keynes) equilibrated in PDGF binding buffer was added. Incubation was continued for 30 min at 20°C with mixing by end over end inversion on a rotating mixer. The affinity matrix was spun down in a microcentrifuge for 2 min and the supernatant removed by
25 aspiration. Non-specifically bound ^{125}I -PDGF-BB was removed by resuspension of the pellet in 0.5ml PDGF

binding buffer, mixing by rotation for 5 min, centrifugation and aspiration of the supernatant, followed by two further washes with 0.5ml 0.1% BSA, 0.2% Triton-X-100. The pellet finally obtained was resuspended in 100ul PDGF binding buffer and counted in a Packard gamma counter. For displacement studies, unlabelled PDGF-BB (Amersham International) was added to the stated concentration for the incubation of ¹²⁵I-PDGF-BB with phage.

¹²⁵I-PDGF-BB bound to the fd h-PDGFB-R phage and was immunoprecipitated in this assay. Specific binding to receptor phage was 3.5 to 4 times higher than the non-specific binding with vector phage fdTPs/Bs or no phage (fig 19). This binding of ¹²⁵I-PDGF-BB could be displaced by the inclusion of unlabelled PDGF-BB in the incubation with phage at 37°C (fig 20). At 50nM, unlabelled PDGF-BB the binding of ¹²⁵I-PDGF-BB was reduced to the same level as the fdTPs/Bs and no phage control. Figure 21 shows the same data, but with the non-specific binding to vector deducted.

These results indicate that a specific saturable site for ¹²⁵I-PDGF-BB is expressed on fd phage containing cloned h-PDGFB-R DNA. Thus, the phage can display the functional extracellular domain of a cell surface receptor.

Example 19, Construction of Binding molecule-Gene III
Phagemid

It would be useful to improve the transfection efficiency of the phage-binding molecule system and also
5 to have the possibility of displaying different numbers and specificities of binding molecules on the surface of the same bacteriophage. The applicants have devised a method that achieves both aims.

The approach is derived from the phagemid system
10 based on pUC119 [Vieira, J and Messing, J. (1987) Methods Enzymol. 153:3]. In brief, gene III from fd-CAT2 (example 5) and fd-CAT2 ScFv D1.3 (example 2) was cloned downstream of the lac promoter in pUC119 in order that the mutated gene III could be 'rescued' by M13K07 helper
15 phage [Vieira, J and Messing, J. et supra.]. The majority of rescued phage would be expected to contain a genome derived from the pUC119 plasmid that contains the binding molecule-gene III fusion and should express varying numbers of the binding molecule on the surface up
20 to the normal maximum of 3-5 molecules of gene III on the surface of wild type phage. The system has been exemplified below using an antibody as the binding molecule.

An fdCAT2 containing the single chain Fv form of the
25 D1.3 antilysozyme antibody was formed by digesting FDTSCFVD1.3 (example 2) with PstI and XhoI, purifying the

fragment containing the scFv fragment and ligating this into PstI and XhoI digested fdCAT2. The appropriate clone, called fdCAT2 scFvD1.3 was selected after plating onto 2xTY tetracyclin (15µg/ml) and confirmed by
 5 restriction enzyme and sequence analysis.

Gene III from fd-CAT2 (example 5) and fd-CAT2 ScFv D1.3 was PCR-amplified using the primers shown below:

Primer A: TGC GAA GCT TTG GAG CCT TTT TTT TTG GAG ATT TTC
 AAC G

10 Primer B: CAG TGA ATT CTT ATT AAG ACT CCT TAT TAC GCA GTA
 TGT TAG C

Primer A anneals to the 5' end of gene III including the ribosome binding site and incorporates a Hind III site. Primer B anneals to the 3' end of gene III at the
 15 C-terminus and incorporates two UAA stop codons and an EcoRI site. One hundred ng of fd-CAT2 and fd-CAT2 ScFv D1.3 DNA was PCR-amplified in a total reaction volume of 50µl as described in example 11, except that 20 cycles of amplification were performed: 94°C 1 minute, 50°C 1
 20 minute, 72°C 3 minutes. This resulted in amplification of the expected 1.2Kb fragment from fd-CAT2 and a 1.8Kb fragment from fd-CAT2 ScFv D1.3.

The PCR fragments were digested with EcoRI and Hind III, gel-purified and ligated into EcoRI- and Hind III-cut and dephosphorylated pUC119 DNA and transformed into
 25 E.coli TG1 using standard techniques (Sambrook et al., et

supra). Transformed cells were plated on SOB agar containing 100µg/ml ampicillin and 2% glucose. The resulting clones were called pCAT-3 (derived from fd-CAT2) and pCAT-3 ScFv D1.3 (derived from fd-CAT2 ScFv D1.3).

Example 20, Rescue of Anti-Lysozyme Antibody Specificity from pCAT-3 ScFv D1.3 by M13K07

Single CAT-3 and CAT-3 ScFv D1.3 colonies were picked into 1.5ml 2YT containing 100µg/ml ampicillin and 2% glucose, and grown 6hrs at 30°C. 30µl of these stationary cells were added to 6mls 2YT containing 100µg/ml ampicillin and 2% glucose in 50ml polypropylene tubes (Falcon, Becton Dickinson Labware, 1950 Williams Drive, Oxnard, CA. USA) and grown for 1.5hrs at 30°C at 380rpm in a New Brunswick Orbital Shaker (New Brunswick Scientific Ltd., Edison House 163 Dixons Hill Road, North Mimms. Hatfield, UK). Cells were pelleted by centrifugation at 5,000g for 25 minutes and the tubes drained on tissue paper. The cell pellets were then resuspended in 6mls 2YTamp containing 100µg/ml ampicillin (no glucose) and 4mls 2YT containing 1.25×10^9 p.f.u. ml⁻¹ M13K07 bacteriophage added. The mixture was left on ice for 5 minutes followed by growth at 35°C for 45 minutes at 450rpm. A cocktail was then added containing 4µl 100µg/ml ampicillin, 0.5µl 0.1M IPTG and 50µl 10mg/ml

Kanamycin, and the cultures grown overnight at 35°C, 450rpm.

The following day the cultures were centrifuged and phage particles PEG precipitated as described in example

5 6. Phage pellets were resuspended in 100µl TE (tris-EDTA see example 6) and phage titred on E.coli TG1. Aliquots of infected cells were plated on 2YT containing either 100µg/ml ampicillin to select for pUC119 phage particles, or 50µg/ml Kanamycin to select for the M13 K07
10 helper phage. Plates were incubated overnight at 37°C and antibiotic-resistant colonies counted:

DNA	amp ^R	Kan ^R
pCAT-3	1.8x10 ¹¹ colonies	1.2x10 ⁹ colonies
pCAT-3ScFv D1.3	2.4x10 ¹¹ colonies	2.0x10 ⁹ colonies

15 ~~This shows that the amp^R phagemid particles are~~ infective and present in the rescued phage population at a 100-fold excess over Kan^R M13K07 helper phage.

Phage were assayed for anti-lysozyme activity by ELISA as described in example 6, with the following
20 modifications:

- 1) ELISA plates were blocked for 3 hrs with 2% Marvel/PBS
- 2) 50µl phage, 400µl 1xPBS and 50µl 20% marvel were mixed end over end for 20 minutes at room temperature
25 before adding 150µl per well.
- 3) Phage were left to bind for 2 hours at room

temperature.

4) All washes post phage binding were:

2 quick rinses PBS/0.5% TWEEN 20

3x2 minute washes -----"

5 2 quick rinses PBS no detergent

3x2 minute washes -----"

The result of this ELISA is shown in figure 22, which shows that the antibody specificity can indeed be rescued efficiently.

10 It is considered a truism of bacterial genetics that when mutant and wild-type proteins are co-expressed in the same cell, the wild-type protein is used preferentially. This is analagous to the above situation wherein mutant (i.e. antibody fusion) and wild-type gene
15 III proteins (from M13K07) are competing for assembly as part of the pUC119 phagemid particle. It is therefore envisaged that the majority of the resulting pUC 119 phage particles will have fewer gene III-antibody fusion molecules on their surface than is the case for the
20 purely phage system described for instance in example 2. Such phagemid antibodies are therefore likely to bind antigen with a lower avidity than fd phage antibodies with three or more copies of the antibody fusion on their surfaces (there is no wild-type gene III protein in the
25 system described, for instance, in example 2), and provide a route to production of phage particles with

different numbers of the same binding molecule (and hence different avidities for the ligand/antigen) or multiple different binding specificities on their surface, by using helper phage such as M13K07 to rescue cells
5 expressing two or more gene III-antibody fusions.

It is also possible to derive helper phage that do not encode a functional gene III in their genomes (by for example deleting the gene III sequence or a portion of it or by incorporating an amber mutation within the gene).
10 These defective phages will only grow on appropriate cells (for example that provide functional gene III in trans, or contain an amber suppressor gene), but when used to rescue phage antibodies, will only incorporate the gene III antibody fusion encoded by the phagemid into
15 the released phage particle.

Example 21. Transformation Efficiency of pCAT-3 and pCAT-3 ScFv D1.3 phagemids

PUC 19, pCAT-3 and pCAT-3 ScFv D1.3 plasmid DNAs,
20 and fdCAT-2 phage DNA was prepared, and used to transform E.coli TG1, pCAT-3 and pCAT-3 ScFv D1.3 transformations were plated on SOB agar containing 100µg/ml ampicillin and 2% glucose, and incubated overnight at 30°C. fdCAT-2 transformations were plated on 2YT agar containing
25 15µg/ml tetracycline and incubated overnight at 37°C. Transformation efficiencies are expressed as colonies per

µg of input DNA.

		DNA	Transformation efficiency
		pUC 19	1.10^9
5		pCAT-3	1.10^8
		pCAT-3ScFv D1.3	1.10^8
		fd CAT-2	8.10^5

As expected, transformation of the phagemid vector is approximately 100-fold more efficient than the parental fdCAT-2 vector. Furthermore, the presence of a ScFv antibody fragment does not compromise efficiency. This improvement in transformation efficiency is practically useful in the generation of phage antibody libraries that have large repertoires of different binding specificities.

Table 1. Enrichment of pAb (D1.3) from vector population

INPUT RATIO ^a	OUTPUT RATIO		ENRICHMENT ^d
	oligo ^b	ELISA ^c	
pAb : fd-CAT1	pAb : total phage	pAb : total phage	
Single Round			
1 : 4×10^3	43/124		1.3×10^3
1 : 4×10^4	2/82		1.0×10^3
Two Rounds			
1 : 4×10^4	197/372		2.1×10^4
1 : 4×10^5	90/356	3/24	1.0×10^5
1 : 4×10^6	27/183	5/26	5.9×10^5
1 : 4×10^7	13/278		1.8×10^6

Footnotes: ^aApproximately 10^{12} phage with the stated ratio of pAb (D1.3) : FDTPs/Bs were applied to 1 ml lysozyme-sepharose columns, washed and eluted. ^bTG1 cells were infected with the eluted specific binding phage and plated onto TY-tet plates. After incubation overnight at 30-37°C the plates were analysed by hybridisation to the ^{32}P -labelled oligonucleotide VH1FOR (Ward et al op cit) which is specific to pAb D1.3. ^cSingle colonies from overnight plates were grown, phage purified, and tested for lysozyme binding. ^dEnrichment was calculated from the oligonucleotide probing data.

Table 2 Enrichment of pAb(D1.3) from mixed pAb population

Input Ratio ¹ (pAbD1.3:pAbNQ11)	Output Ratio ² (pAb D1.3: Total phage)	Enrichment
Single Round		
1 : 2.5×10^4	18/460	0.98×10^3
1 : 2.5×10^5	3/770	0.97×10^3
1 : 2.5×10^6	0/112	
pAb NQ11 only	0/460	
Second Round		
1 : 2.5×10^4	119/170	1.75×10^4
1 : 2.5×10^5	101/130	1.95×10^5
1 : 2.5×10^6	102/204	1.26×10^6
1 : 2.5×10^7	0/274	
1 : 2.5×10^8	0/209	
pAb NQ11 only	0/170	

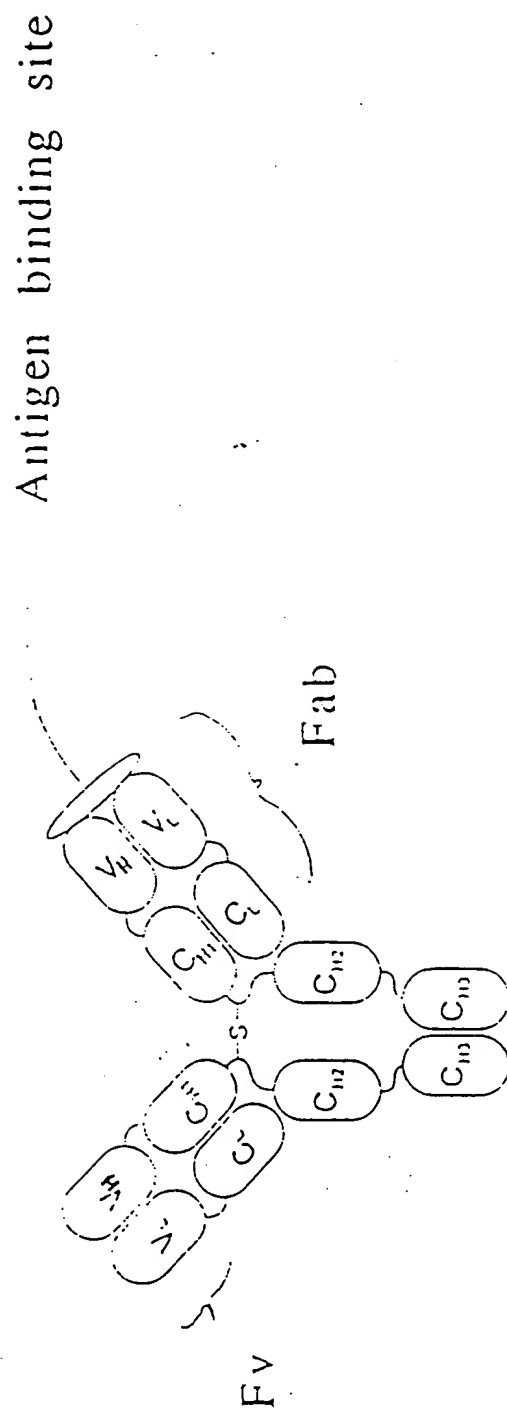
Notes

1. 10^{10} phage applied to a lysozyme column as in table 1.
2. Plating of cells and probing with oligonucleotide as in table 1, except the oligonucleotide was D1.3CDR3A

Table 3: Enzymic activity of phage-enzyme

Input	ng of enzyme or No. of phage	Rate (OD/hr)	No. of molecules of Enzyme equivalent ($\times 10^{-11}$)
Pure Enzyme	335	34	24.5
Pure Enzyme	177.5	17.4	12.25
Pure Enzyme	88.7	8.7	6.125
Pure Enzyme	44.4	4.12	3.06
Pure Enzyme	22.2	1.8	1.5
Pure Enzyme	11.1	0.86	0.76
No Enzyme	0	0.005	0
fd-phoA1/TG1	1.83×10^{11}	5.82	4.2
fd-CAT2/TG1	1.0×10^{12}	0.155	0.112
fd-phoA1/KS272	7.1×10^{10}	10.32	7.35
fd-CAT2/KS272	8.2×10^{12}	0.038	0.027

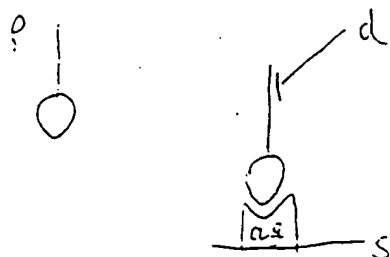
FIGURE 1: Antibody structure



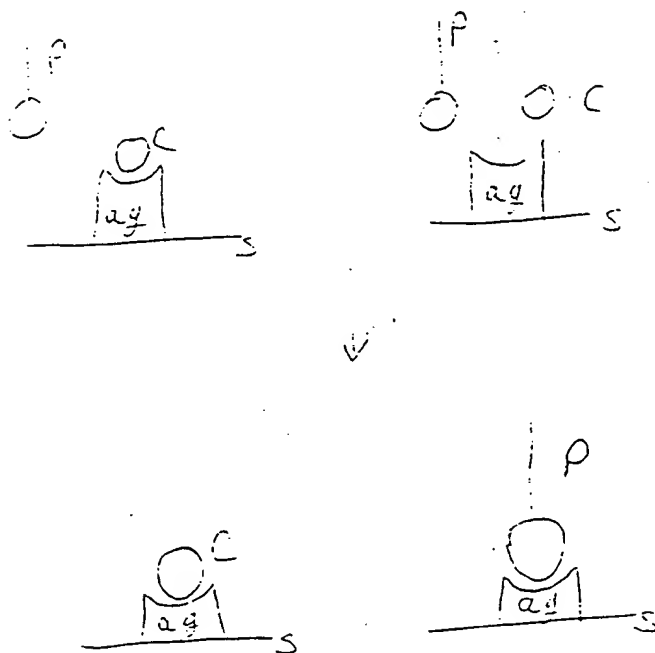
The single domain antibody (dAb, Ward et al. 1989) consists of a single V_H domain.

FIGURE 2: ASSAY FORMATS

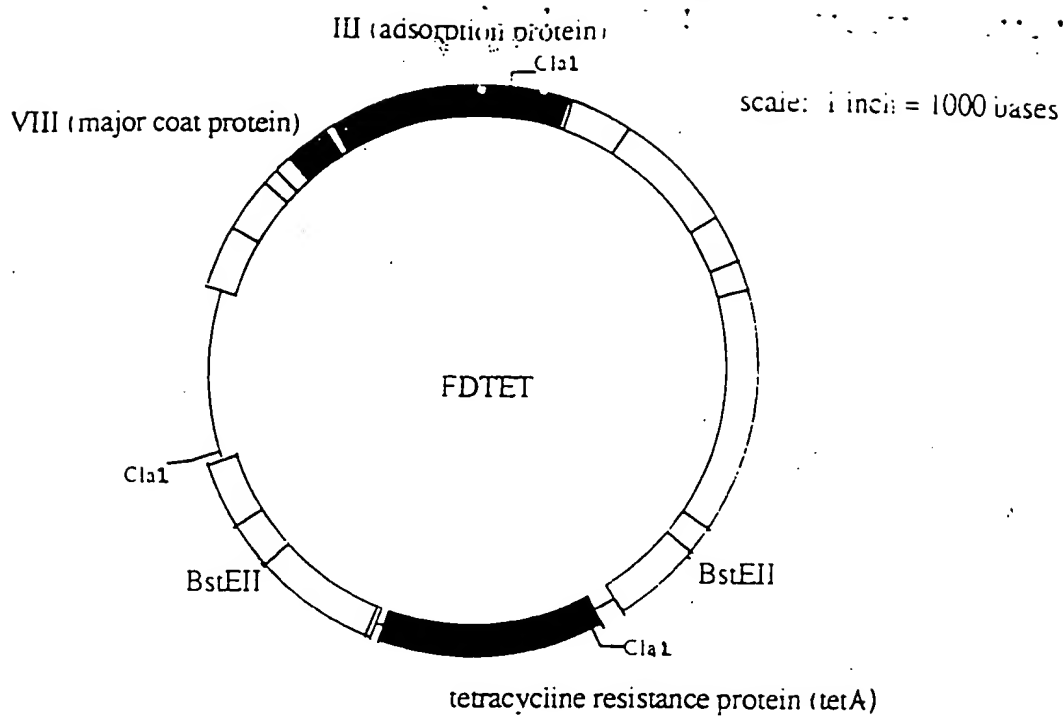
2 i) Binding/elution



2 ii) Competition



- P - Phage antibody population to be sampled.
- ag - Antigen to which binding required.
- c - Competitor antibody/ phAb/ligand etc population.
- s - Surface (eg plastic, beads etc).
- d - Detection system



fd-tet



cleave with BstEII



fill in with Klenow



re-ligate



FDT δ Bst



in vitro mutagenesis (oligo 1)



FDTPs/Bs



in vitro mutagenesis (oligo 2)



FDTPs/Xh

Figure 3 Scheme for construction of vectors

1

(1653)
 Oligo 1 ACA ACT TTC AAC AGT TGA GGA GAC GGT GAC CGT AAG CTT CTG CAG TTG GAC CTG AGC
 GGA GTG AGA ATA (1620)

(1653)
 Oligo 2 ACA ACT TTC AAC AGT TTC CCG TTT GAT CTC GAG CTC CTG CAG TTG GAC CTG

(1704)
 Oligo 3 GTC GTC TTT CCA GAC GTT AGT

2

GENE III

GENE III

SIGNAL
 CLEAVAGE SITE

(1624)
 A TCT CAC TCC GCT -----

(1650)
 GAA ACT GTT GAA AGT

Q V Q L Q V T V S S
 B TCT CAC TCC GCT CAG GTC CAA CTG CAG AAG CTT ACG GTC ACC GTC TCC TCA ACT GTT GAA AGT
 PstI BstEII

Q V Q L Q L E I K R
 C TCT CAC TCC GCT CAG GTC CAA CTG CAG GAG CTC GAG ATC AAA CGG GAA ACT GTT GAA AGT
 PstI XhoI

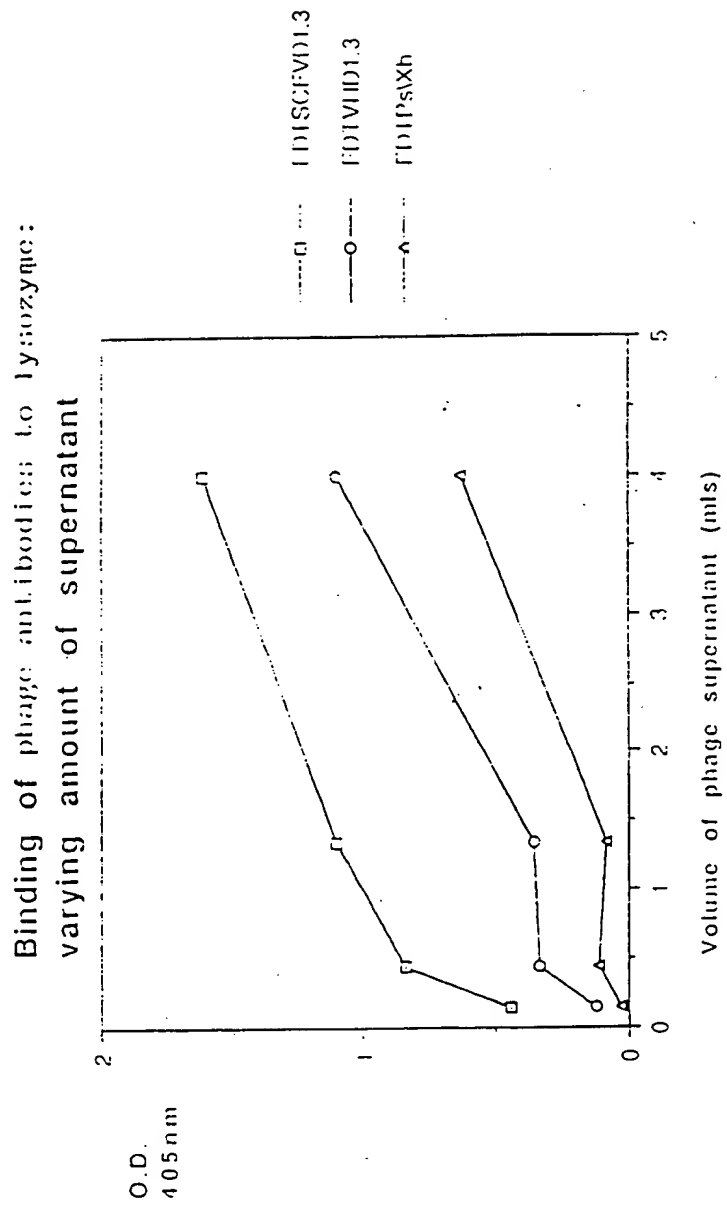
B = FDTPs / Bs

C = FDTPs / Xh

Figure 4. Sequence of oligos and vectors

Σcore:

Fig. 6.



Methods as described in example 4

Binding of phage antibodies to lysozyme:
effect of coating concentration

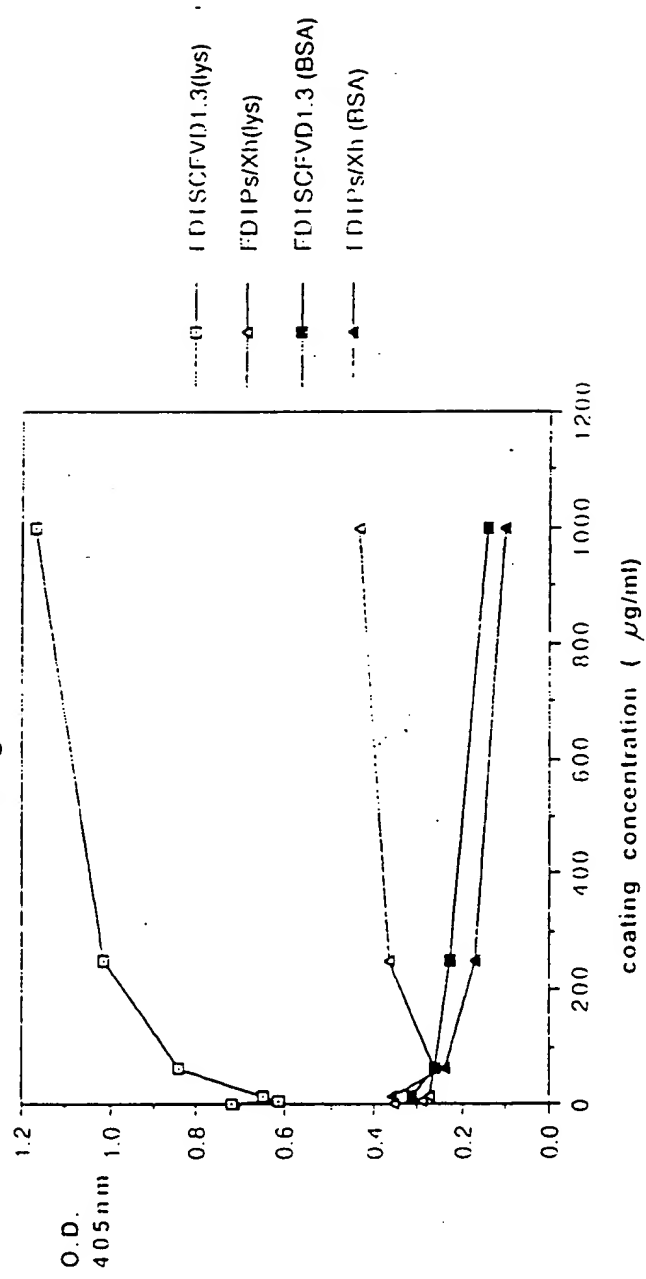
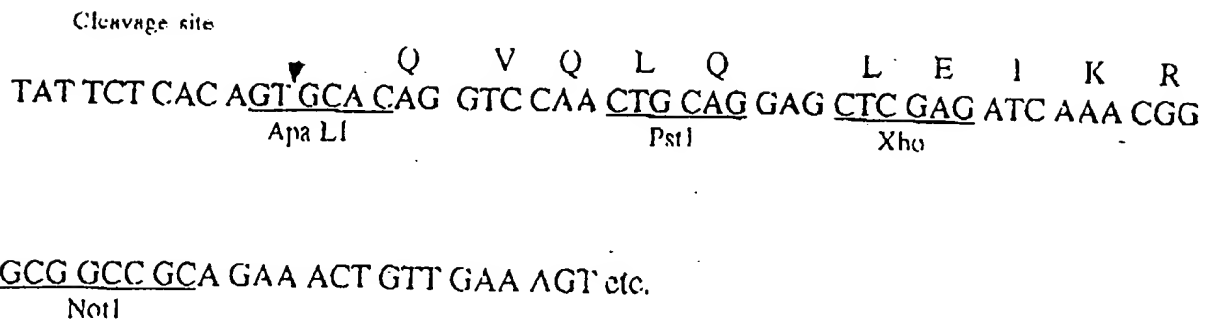


Fig. 7

Methods as described in example 4

Fig. 8. Sequence around the cloning site of
fd-CAT2.



Restriction enzyme sites are shown as well as the amino acids encoded by antibody derived sequences. These are flanked at the 5' end by the gene 3 signal peptide and at the 3' end by 3 alanine residues (encoded by the Not I restriction site) and the remainder of the mature gene 3 protein.

9/25

Figure 9 Binding of pAb(D1.3) to lysozymes

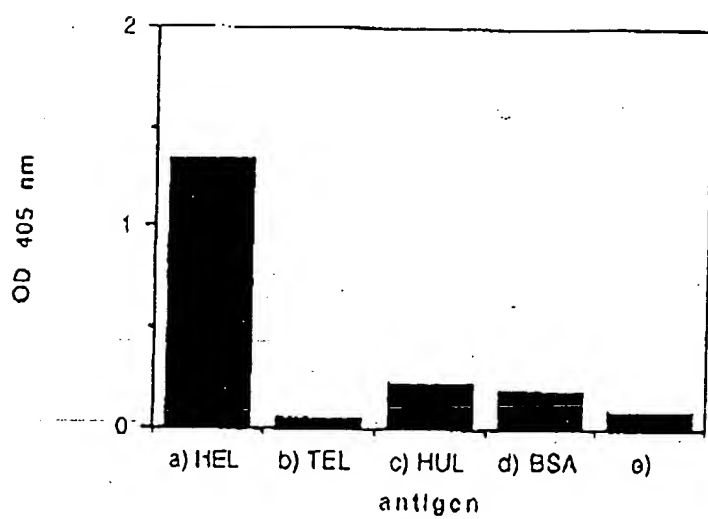


Figure 10: S quence f Fab D1.3

M K Y L L P T A A A G L L L P A A Q P A
 CATGCAAATTCATTTCAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACCTGCTGCCCCAACCAG
 11 21 31 41 51 61 71 81 91

M A Q V Q L Q E S G P G L V A P S Q S L S I T C T V S G F S
 CGATGGCCCCAGGTGCAGCTGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCATCACATGCACCGTCTCAGGGTTCT
 101 111 121 131 141 151 161 171 181

L T G Y G V N W V R Q P P G K G L E W L G M I W G D G N T D
 CATTAAACCGCTATGGTGTAAACTGGGTTCGCCAGCCTCCAGGAAAGGGTCTGGAGTGGCTGGGAATGATTTGGGGTGATGGAAACACAG
 191 201 211 221 231 241 251 261 271

Y H S A L K S R L S I S K D N S K S Q V F L K M N S L H T D
 TATAATTCAGCTCTCAAATCCAGACTGAGCATCAGCAAGGACAACTCCAAGAGCCAAGTTTCTTAAAAATGAACAGTCTGCACACTG
 281 291 301 311 321 331 341 351 361

D T A R Y Y C A R E R D Y R L D Y W G Q G T T V T V S S A S
 ATGACACAGCCAGGTACTACTGTGCCAGAGAGAGAGATTATAGGCTTGACTACTGGGGCCAAGGCACCAACGGTCACCGTCTCCTCAGCCT
 371 381 391 401 411 421 431 441 451

T K G P S V F P L A P S S K S T S G G T A A L G C L V K D Y
 CCACCAAGGGGCCCATCGGTCTTCCCCCTGGCACCCCTCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACT
 461 471 481 491 501 511 521 531 541

F P E P V T V S W H S G A L T S G V H T F P A V L Q S S G L
 ACTTCCCCGAACCGGTGACGGTGTCTGTGGAACCTCAGGCGCCCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCTCCTCAGGAC
 551 561 571 581 591 601 611 621 631

Fig. 10. (cont.) (1)

: : 11/25 :

Y S L S S V V T V P S S S L G T Q T Y I C N V H H N P S N T
TACTCCCTCAGCAGCGTGGTGACTGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAACCCCAAGCAACA
641 651 661 671 681 691 701 711 721

K V D K K V E P K S S * * M K
CCAAGGTCGACAAGAAAGTTGAGCCCAAATCTTCATAATAACCCGGGAGCTTGCATGCAAATCTATTTCAGGAGACAGTCATAATGAA
731 741 751 761 771 781 791 801 811

Y L L P T A A A G L L L P A A Q P A M A D I E L T Q S P A S
TACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACCTGCTGCCCAACCAGCGATGGCCGACATCGAGCTCACCCAGTCTCCAGCCTC
821 831 841 851 861 871 881 891 901

L S A S V G E T V T I T C R A S G N I H N Y L A W Y Q Q K Q
CCTTTCTGCGTCTGTGGGAGAACTGTCAACCATCACATGTGAGCAAGTGGGAATATTACAATTATTTAGCATGGTATCAGCAGAAACA
911 921 931 941 951 961 971 981 991

G K S P Q L L V Y Y T T T L A D G V P S R F S G S G S G T Q
GGGAAATCTCCTCAGCTCCTGGTCTATTATACAAACCTTAGCAGATGGTGTGCCATCAAGGTTCAAGTGGCAGTGGATCAGGAACACA
1001 1011 1021 1031 1041 1051 1061 1071 1081

Y S L K I N S L Q P E D F G S Y Y C Q H F W S T P R T F G G
ATATTCTCTCAAGATCAACAGCCTGCAGCCTGAAGATTTTGGGAGTTATTACTGTCAACATTTTTGGAGTACTCCTCGGACGTTCCGGTGG
1091 1101 1111 1121 1131 1141 1151 1161 1171

G T K L E I K R T V A A P S V F I F P P S D E Q L K S G T A
AGGCACCAAGCTCGAGATCAACCGGACTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAAGTGC
1181 1191 1201 1211 1221 1231 1241 1251 1261

Fig. 10. cont. (2). 12/25

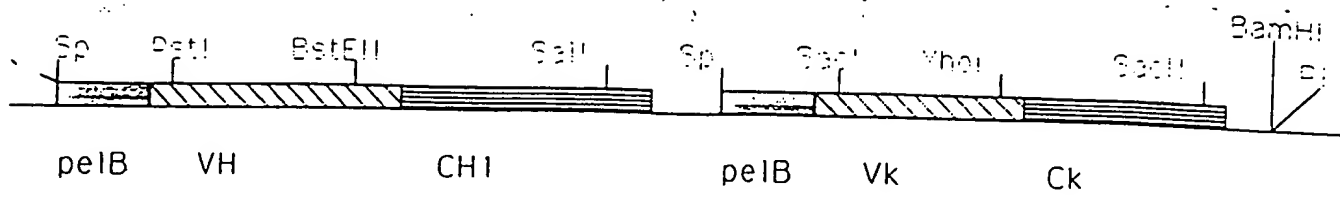
S V V C L L N N F Y P R E A K V Q W K V D N A L Q S G N S Q
CTCTGTTGTTGCTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCA
1271 1281 1291 1301 1311 1321 1331 1341 1351

E S V T E Q D S K D S T Y S L S S T L T L S K A D Y E K H K
GGAGAGTGTTCACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAA
1361 1371 1381 1391 1401 1411 1421 1431 1441

V Y A C E V T H Q G L S S P V T K S F N R G E S * *
AGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGTTGCGCCGTCACAAAGAGCTTCAACGCGGAGAGTCATAGTAAGGATCCAGCTC
1451 1461 1471 1481 1491 1501 1511 1521 1531

GAATTC

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FabD1.3 in
pUC19

Fig. 10 cont. (3).

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Fig. 11 Comparison of lysozyme-binding by phage-Fab and phage -ScFv

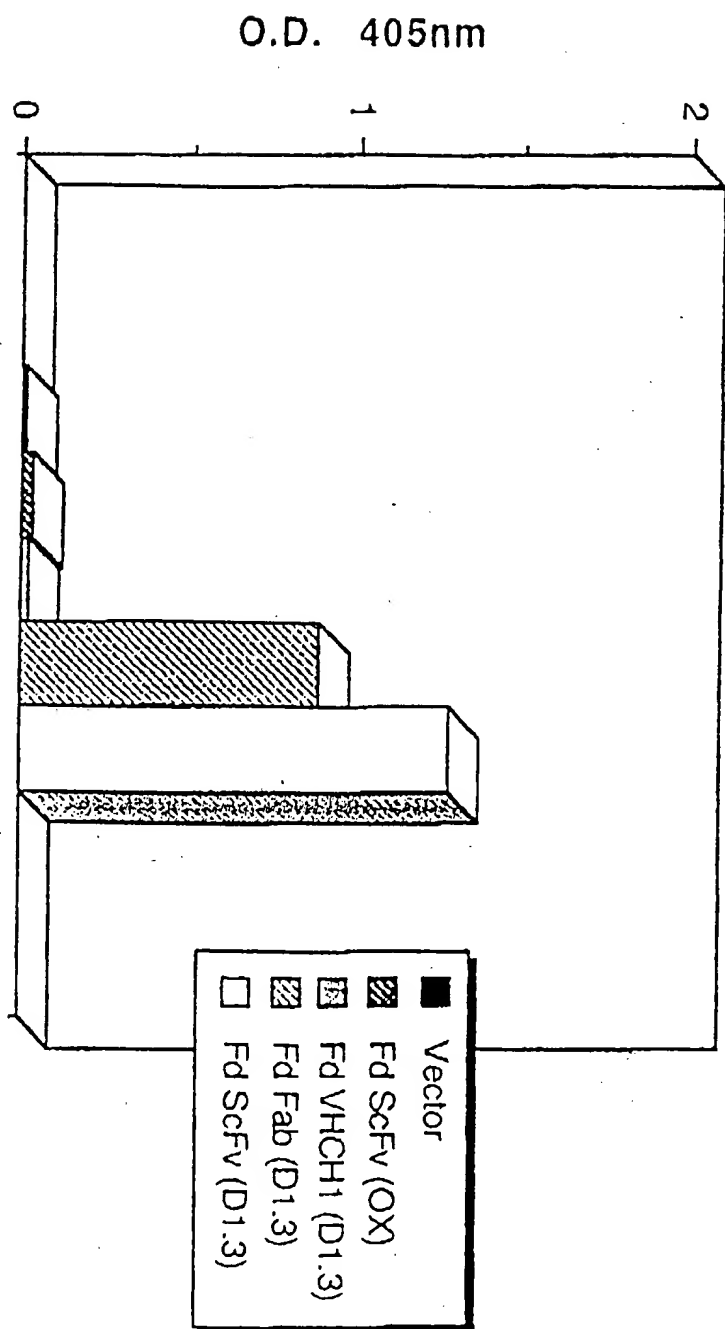


Fig. 12

B

A



Figure 13. Sequence of pNOF1SuFv

Q V Q L O E S G G G L V Q P G G
 CAG GTG CAG CTG CAG GAG TCA GGA GGA GGC TTG GTA CAG CCT GGG GGT
 PstI
 S L R L S C A T S G F T F S N Y
 TCT CTG AGA CTC TCC TGT GCA ACT TCT GGG TTC ACC TTC AGT AAT TAC
 Y M G W V R Q P P G K A L E W L
 TAC ATG GGC TGG GTC CGC CAG CCT CCA GGA AAG GCA CTT GAG TGG TTG
 G S V R N K V N G Y T T E Y S A
 GGT TCT GTT AGA AAC AAA GTT AAT GGT TAC ACA ACA GAG TAC AGT GCA
 S V K G R F T I S R D N F Q S I
 TCT GTG AAG GGG CGG TTC ACC ATC TCC AGA GAT AAT TTC CAA AGC ATC
 L Y L Q I N T L R T E D S A T Y
 CTC TAT CTT CAA ATA AAC ACC CTG AGA ACT GAG GAC AGT GCC ACT TAT
 Y C A R G Y D Y G A W F A Y W G
 TAC TGT GCA AGA GGC TAT GAT TAC GGG GCC TGG TTT GCT TAC TGG GGC
 Q G T L V T v s s g g g g s g g g s
 CAA GGG ACC CTG GTC ACC gtc tcc tca ggtggaggcggttcaggcggaggtggctct
 BstEII
 g g g g s d i E L T Q T P L S L P V
 ggcggtggcggtacgac atc GAG CTC ACC CAA ACT CCA CTC TCC CTG CCT GTC
 SacI
 S L G D Q A S I S C R S S Q S I
 AGT CTT GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC ATT
 V H S N G N T Y L E W Y L Q K P
 GTA CAT AGT AAT GGA AAC ACC TAT TTA GAA TGG TAC CTG CAG AAA CCA
 PstI
 G Q S P K L L I Y K V S N R F S
 GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT
 G V P D R F S G S G S G T D F T
 GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCG GGG ACA GAT TTC ACA
 L K I S R V E A E D L G V Y Y C
 CTC AAG ATC AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TAC TGC
 F Q G S H V P Y T F G G G T K L
 TTT CAA GGT TCA CAT GTT CCG TAC ACG TTC GGA GGG GGG ACC AAG CTC
 E I K R
 GAG ATC AAA CGG
 XhoI

Restriction sites referred to in the text are shown underlined. The sequence contributed by the linker is shown in lower case.

Fig. 15. Sequence surrounding phoA insertion in fd-phoA1

SEQUENCE AT 5' END OF phoA INSERTION IN fd-phoA1

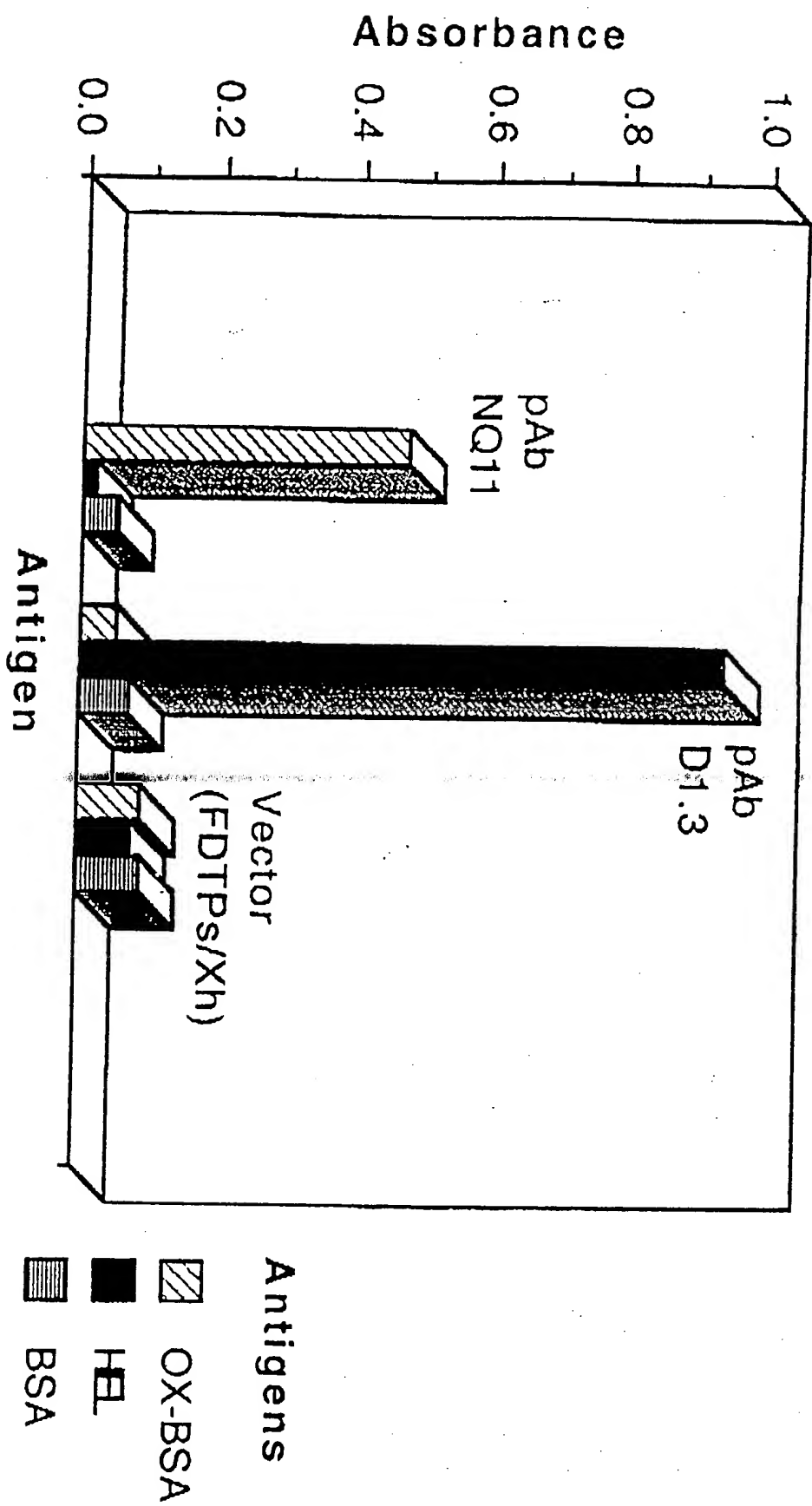
Signal peptide
cleavage site
▼
TCT CAC AGT GCA CAA ACT GTT GAA CGG ACA CCA GAA ATG CCT GTT CTG
ApaI R T P E M P V L

SEQUENCE AT 3' END OF phoA INSERTION IN fd-phoA1

K A A L G L K
AAA GCC GCT CTG GGG CTG AAA GCG GCC GCA GAA ACT GTT GAA AGT etc.
NotI

The restriction sites used for cloning are shown as well as the amino acids encoded by phoA around the insertion site. In this example, the first five amino acids of the mature fusion will actually come from gene 3.

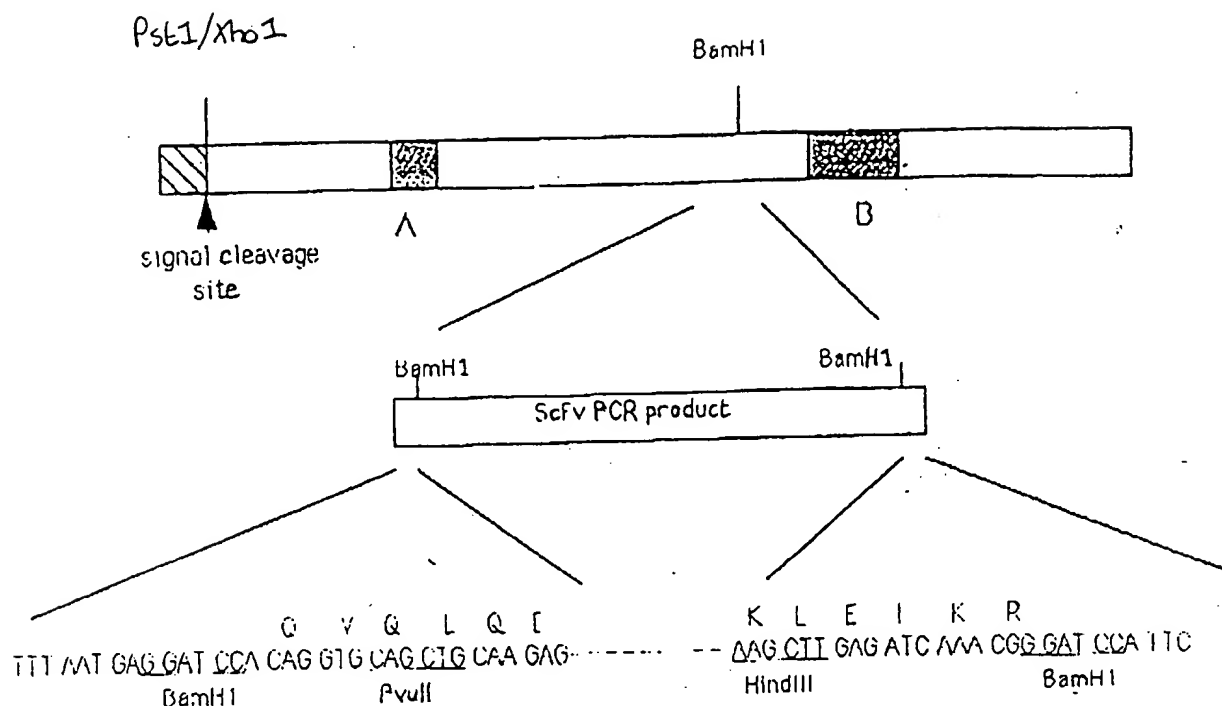
Figure 14. Binding of pAbs to specific antigens



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Figure 16. Structure of gene 3

1)



2)

A

(1834) 5' GAG GGT GGT GGC TCT
 " " "C " "
 " " "C " "
 " " "C " ACT 3'(1839)

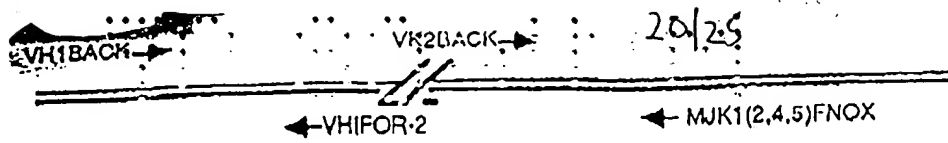
B

(2284) 5' - GGC GGC GGC TCT
 - GGT GGT GGT "
 - " GGC GGC "
 GAG " " GGC "
 " " " GGT "
 " " " GGC "
 " " " GGT "
 " " " GGC " 3'(2379)

verse complement of mutagenic
 go G3Bamlink

5' GAG GGT GGC GGA TCC
 T
 GAG GGT GGC GG 3'

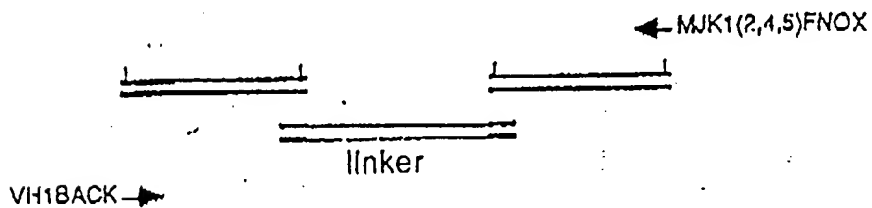
ring is according to Beck et al (1978, supra).



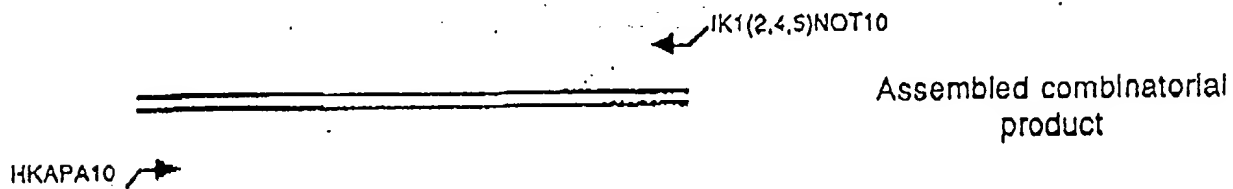
PRIMARY PCR FROM cDNA



MIX PRODUCTS WITH LINKER DNA
(MADE BY PCR)



SECOND ASSEMBLY PCR
USING 'OUTER' PRIMERS



PULL-THROUGH (tagged primers)



DIGEST AND CLONE

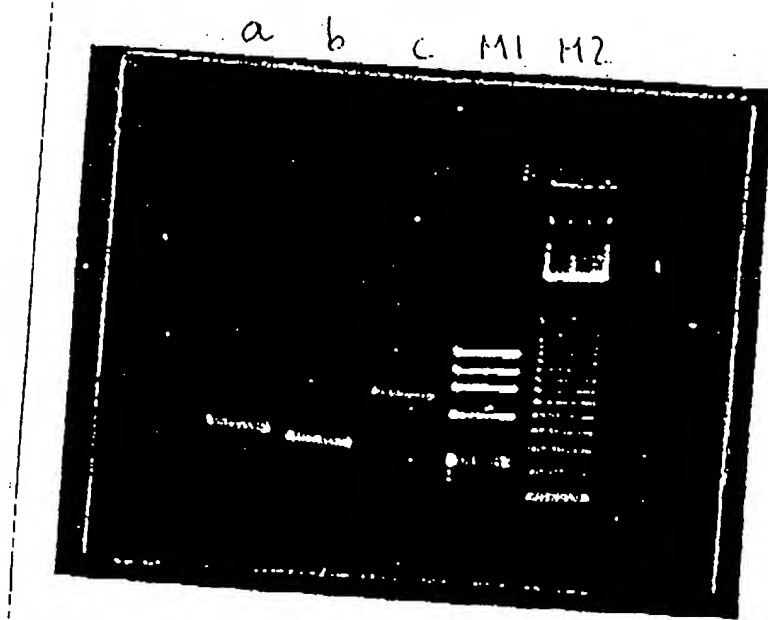
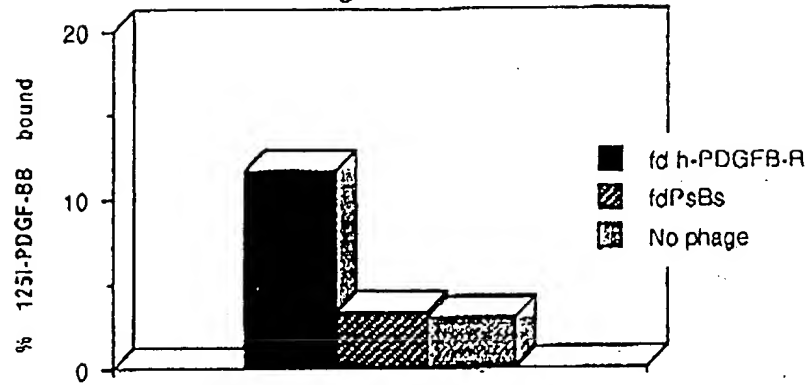


Fig. 18

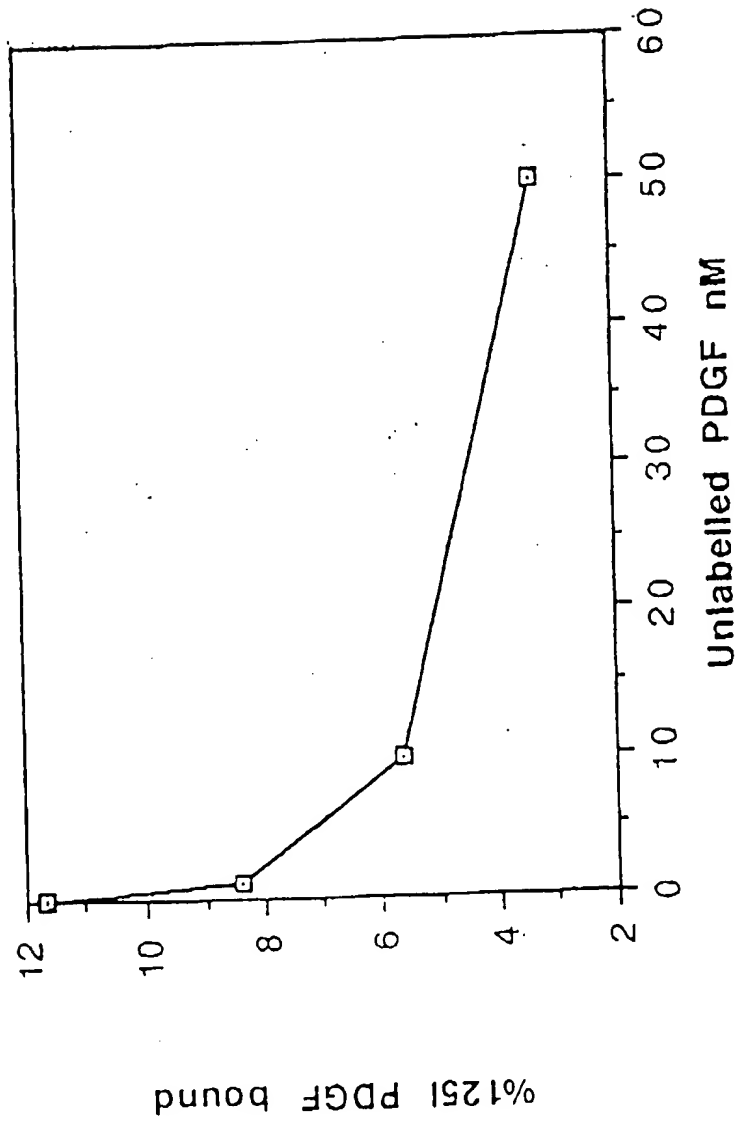
22/25

Figure 19



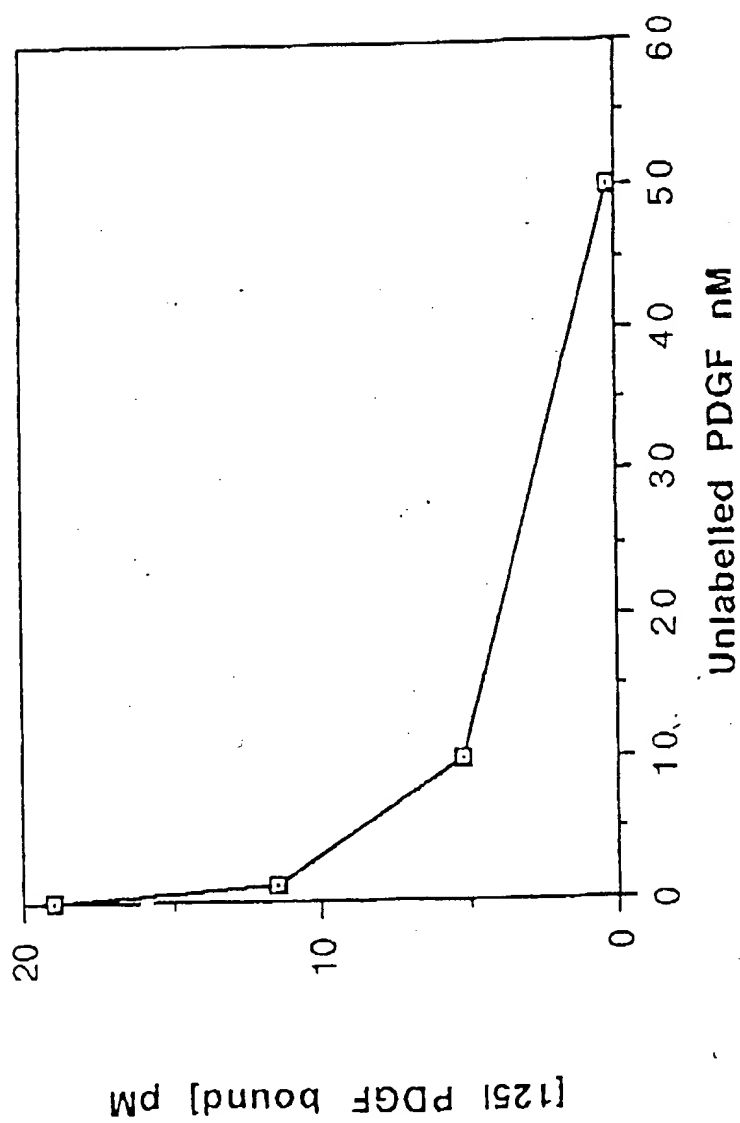
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Figure 20 Displacement by unlabelled PDGF



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Figure 21 PGDF displacement vector binding deducted



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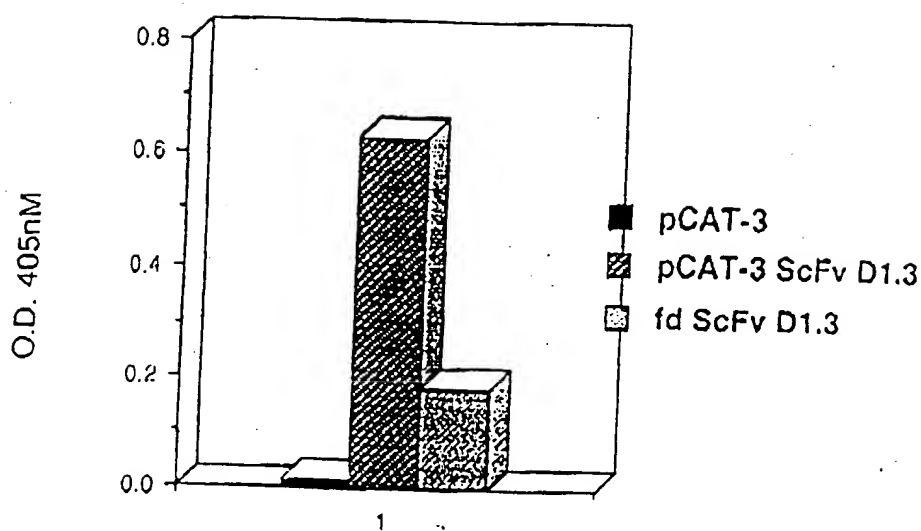


Figure 22: Elisa of lysozyme binding by pCAT-3 ScFv D1.3 phagemid in comparison with pCAT-3 vector (both rescued by M13KO7) and fdCAT2 ScFvD1.3 as described in example 17. ELISA was performed as described in example 6 with modifications detailed in example 17.

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APPLICANTS: O'BRIEN, Caroline Jane
Mansfield House
Wolvershill Road
Banwell
Avon

TITLE: BINDING SUBSTANCES

INVENTORS: CHISWELL, David John
1 Sandhill House
Middle Claydon
Bucks MK18 2LD

MCCAFFERTY, John
36 Woodlands Park Road
London N15

DERIVATION OF RIGHT TO APPLY:

PRIORITY CLAIM: FIRST FILING

MEWBURN ELLIS

CHARTERED PATENT AGENTS · EUROPEAN PATENT ATTORNEYS

152 Cambridge Science Park, Milton Road, Cambridge CB4 4GG
Offices at: Bristol, London, Manchester, Munich, Newcastle, Sheffield

BINDING SUBSTANCES

The present invention relates to binding substances. The present invention also relates to methods for the production of binding substances eg binding molecules and to the biological binding molecules produced by these methods. The present invention also relates to: a) the production of antibodies, receptor molecules and fragments and derivatives of these antibodies and receptor molecules; b) viruses encoding the above identified molecules, which viruses have the ability to present said molecules at their surfaces; c) packages comprising a virus and an above identified molecule presented at the viral surface; and d) screening techniques utilising the unique properties of these packages.

Owing to their high specificity for a given antigen, the advent of monoclonal antibodies (Kohler, G. and Milstein C; 1975 Nature 256: 495) represented a significant technical break-through with important consequences both scientifically and commercially.

Monoclonal antibodies are made by establishing an immortal mammalian cell line which is derived from a single immunoglobulin producing cell secreting one form of a biologically functional antibody molecule with a particular specificity. Because the antibody-secreting mammalian cell line is immortal, the characteristics of the antibody are reproducible from batch to batch. The key properties of monoclonal antibodies are their specificity for a particular antigen and the reproducibility with which they can be manufactured.

Structurally, the simplest antibody (IgG) comprises four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulphide bonds (see figure 1). Each chain has a constant region (C) and a variable region (V). The antibody has two arms (the Fab region) each of which has a V_L and a V_H region associated with each other. It is this pair of V regions (V_L and V_H) that differ

from one antibody to another, and which together are responsible for recognising the antigen. In even more detail, each V region is made up from three complementarity determining regions (CDR) separated by four framework regions (FR). The CDR's are the most variable part of the variable regions, and they perform the critical antigen binding function. The CDR regions are derived from many potential germ line sequences via a complex process involving recombination, mutation and selection.

It has been shown that the function of binding antigens can be performed by fragments of a whole antibody. Binding fragments are the F_V fragment which comprises the V_L and V_H of a single arm of the antibody, and the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989); which consists of a single heavy chain variable domain (V_H).

Although the F_V fragment is coded for by separate genes, it has proved possible to construct a linker that enables them to be made as a single protein chain (known as single chain F_V (scFv); Bird, R.E. et al., Science 423, 423-426 (1988) Huston, J.S. et al., Proc. Natl. Acad. Sci., USA 85, 5879-5883 (1988)) by recombinant methods.

Whilst monoclonal antibodies, their fragments and derivatives have been enormously advantageous, there are nevertheless a number of limitations associated with them.

Firstly, the therapeutic applications of monoclonal antibodies produced by human immortal cell lines holds great promise for the treatment of a wide range of diseases (Clinical Applications of Monoclonal Antibodies. Edited by E. S. Lennox. British Medical Bulletin 1984. Publishers Churchill Livingstone). Unfortunately, immortal antibody-producing human cell lines are very difficult to establish and they give low yields of antibody (approximately 1 µg/ml). In contrast, equivalent rodent cell lines yield high amounts of antibody (approximately 100 µg/ml). However, the repeated administration of these foreign rodent proteins to humans can lead to harmful hypersensitivity reactions. In the main therefore, these

rodent-derived monoclonal antibodies have limited therapeutic use.

Secondly, a key aspect in the isolation of monoclonal antibodies is how many different antibody producing cells with different specificities, can be sampled, compared to how many need to be sampled in order to isolate a cell producing antibody with the desired specificity characteristics (Milstein, C., Royal Soc. Croonian Lecture, Proc. R. Soc. London B. 239; 1-16, (1990)). For example, the number of different specificities expressed at any one time by lymphocytes of the murine immune system is thought to be approximately 10^7 and this is only a small proportion of the potential repertoire of specificities. However, during the isolation of a typical antibody producing cell with a desired specificity, the investigator is only able to sample 10^3 to 10^4 individual specificities. The problem is worse in the human, where one has approximately 10^{12} lymphocyte specificities, with the limitation on sampling of 10^3 or 10^4 remaining.

This problem has been alleviated to some extent in laboratory animals by the use of immunisation regimes. Thus, where one wants to produce monoclonal antibodies having a specificity against a particular epitope, an animal is immunised with an immunogen expressing that epitope. The animal will then mount an immune response against the immunogen and there will be a proliferation of lymphocytes which have specificity against the epitope. Owing to this proliferation of lymphocytes with the desired specificity, it becomes easier to detect them in the sampling procedure. However, this approach is not successful in all cases, as a suitable immunogen may not be available. Furthermore, where one wants to produce human monoclonal antibodies (eg for therapeutic administration as previously discussed) such an approach is not practically or ethically feasible.

In the last few years, these problems have in part,

been addressed by the application of recombinant DNA methods to the isolation and production of antigen binding fragments of an antibody molecule in bacteria such as E.coli. Furthermore, the use of polymerase chain reaction (PCR) amplification (Saiki, R.K., et al., Science 239, 4387-491 (1988)) to isolate antibody producing sequences from cells and organs, has great potential for speeding up the timescale under which specificities can be isolated. Amplified V_H and V_L genes are cloned directly into vectors for expression in bacteria or mammalian cells (Orlandi, R., et al., 1989, Proc. Natl. Acad. Sci., USA 86, 3833-3837; Ward, E.S., et al., 1989 supra; Larrick, J.W., et al., 1989, Biochem. Biophys. Res. Commun. 160, 1250-1255; Sastry, L. et al., 1989, Proc. Natl. Acad. Sci., USA., 86, 5728-5732). Conversely, some of these techniques can exacerbate the screening problems. For example, large separate heavy and light chain libraries have been produced from immunized mice and combined together in a random combinatorial manner prior to screening (Huse, W.D. et al., 1989, Science 246, 1275-1281). Crucially however, the information held within each cell, namely the specific combination of one light chain with one heavy chain, is lost. This loses most, if not all, of the advantage gained by using immunization protocols in the animal. Currently, only libraries derived from single heavy chain variable domains (dAbs; Ward, E.S., et al., 1989, supra.) do not suffer this drawback, but because not all antibody heavy chain variable regions are capable of binding antigen, more have to be screened.

In addition, the problem of directly screening many different specificities in prokaryotes remains to be solved.

Thus, there is a need for a screening system which ameliorates or overcome one or more of the above or other problems. The ideal system would allow the sampling of very large numbers of specificities (eg of the order of 10^6 and higher) rapid sorting at each cloning round, and rapid

transfer of the genetic material coding for the binding molecule from one stage of the production process, to the next stage.

5 The most attractive candidates for this type of screening, would be prokaryotic organisms (because they grow quickly, are relatively simple to manipulate and because large numbers of clones can be created) which express and retain antibody on their surface. It has already been shown that antibody fragments can be secreted through bacterial
10 membranes with the appropriate signal peptide (Skerra, A., and Pluckthun, A., 1988, Science 240, 1038-1040; Better, M. et al., 1988, Science 240, 1041-1043). However, it has not been shown how an antibody or antibody fragment can be held on the bacterial cell surface in a configuration which
15 allows efficient sampling of its antigen binding properties. In large part, this is because the bacterial surface is a complex structure, and in the gram-negative organisms there is an outer wall which further complicates the position.

Bacteriophage make attractive candidates because in
20 general their surface is a much simpler structure, they can be grown easily in large numbers, are amenable to the practical handling involved in many potential mass screening programmes and they carry genetic information for their own synthesis within a small, simple package. The difficulty
25 has been to practically solve the problem of how to use bacteriophages in this manner. For example, a Genex Corporation patent application number PCT/US88/00716 has proposed that the bacteriophage lambda would be a suitable vehicle for the expression of antibody molecules, but no
30 proposals provide a teaching which enables the general idea to be carried out. For example PCT/US88/00716 does not demonstrate that any sequences: a) have been expressed as a fusion with gene V; b) have been expressed on the surface of lambda; and c) retain biological activity. Furthermore
35 there is no teaching on how to screen for suitable fusions.

The problem of how to use bacteriophages is in fact a

difficult one. The antibody molecule must be inserted into the phage in such a way that the integrity of the phage coat is not undermined, and the antibody itself should be biologically active. Thus the antibody should fold efficiently and correctly and be presented for antigen binding. However, solving the problem for antibody molecules and fragments would also provide a general method for the screening of many receptor molecules.

Surprisingly, the applicants have been able to construct a bacteriophage that expresses and presents on its surface large binding molecules (eg large biologically functional antibody molecules) and which remains intact and infectious. The applicants have called the structure which comprises a virus particle and a binding molecule presented at the viral surface a 'package'. Where the binding molecule is an antibody (or a fragment or derivative of an antibody), the applicants call the package a phage antibody. However, except where the context demands otherwise, where the term phage antibody is used generally it should also be interpreted as referring to any package comprising a virus particle and a binding molecule presented at the viral surface. Phage-antibodies (pAbs) are likely to find a range of applications in screening antibody V-genes encoding antigen binding activities. For example, pAbs could be used in cloning and rescue of hybridomas (Orlandi, R., et al (1989) PNAS 86 p3833-3837), and in the screening of large combinatorial libraries (such as found in Huse, W.D. et al., 1989, Science 246, 1275-1281) In particular, rounds of selection using pAbs may help in rescuing the higher affinity antibodies from the latter libraries. It may be preferable to screen small libraries derived from antigen-selected cells (Casali, P., et al., (1986) Science 234 p476-479) to rescue the original VH/VL pairs comprising the V region of an antibody. The use of pAbs may also allow the construction of entirely synthetic antibodies. For example, V-gene repertoires could be made in vitro by combining unrearranged V genes, with D and J segments. Libraries of

pAbs could then be selected by binding to antigen, hypermutated in the antigen-binding loops in vitro and subjected to further rounds of selection and mutagenesis.

5 The demonstration that a functional antigen-binding site can be expressed on the surface of phage, has implications beyond the construction of novel antibodies. For example, if other protein domains can be expressed at the surface of a phage, phage vectors could be used to clone and select genes by the binding properties of the expressed protein. 10 Furthermore, endless variants of proteins, including epitope libraries built into the surface of the protein, could be made and readily selected for binding activities. In effect other protein architectures might serve as "nouvelle" antibodies. One class of molecules that 15 could be useful in this type of application are receptors. For example, a specific receptor could be expressed on the surface of the phage such that it would bind its ligand. The receptor could then be modified by, for example, in vitro mutagenesis and variants having higher binding 20 affinity for the ligand selected. The screening may be carried out according to one or more of the formats described below with reference to figure 2. Figure 2 refers particularly to phage antibodies. In the situation discussed above the phage antibody is replaced with a phage receptor and the antigen with a ligand l. 25

Alternatively, the receptor-phage could be used as the basis of a rapid screening system for the binding of ligands, altered ligands, or potential drug candidates. The advantages of this system of simple cloning, convenient 30 expression, standard reagents and easy handling makes the drug screening application particularly attractive. In the context of this discussion, receptor means a molecule that binds a specific, or group of specific, ligand(s); the natural receptor could be expressed on the surface of a population of cells, it could be the extracellular domain of 35 such a molecule (whether such a form exists naturally or not), or it could be a soluble molecule performing a natural

binding function in the plasma or within a cell or organ.

Although throughout this application the applicant discuss the possibility of screening for higher affinity variants of phage-antibodies, they recognise that in some applications, for example low affinity chromatography (Ohlson, S. et al Anal. Biochem. 169, p204-208 (1988)), it may be desirable to isolate lower affinity variants.

The system of expressing binding molecules on the phage surface could also be used as a primary cloning system. For example, a cDNA library could be constructed and inserted into the bacteriophage and this library screened for the ability to bind a ligand. The ligand/binding molecule combination could include receptor/ligand, enzyme/substrate (or analogue), nucleic acid binding protein/nucleic acid etc. This could be a preferred method to isolate a clone of either molecule in the pair, if the other molecule was available.

Targeted gene transfer

20

A useful and novel set of applications makes use of the binding protein on the phage to target the phage genome to a particular cell or group of cells. For example, a phage-antibody specific for a cell surface receptor could be used to bind to the target cell surface. The phage could then be internalised, either through the action of the receptor itself or as the result of another event (eg. an electrical discharge such as in the technique of electroporation). The phage genome would then be expressed if the relevent control signals (for transcription and translation and possibly replication) were present. This would be particularly useful if the genome contained a sequence whose expression was desired in the target cell (along with the appropriate expression control sequences). A useful sequence might confer antibiotic resistance to the recipient cell or label the cell by the expression of its product (eg. if the sequence expressed a detectable gene product such as a

35

luciferase, see White, M, et al, Techniques 2(4), p194-201 (1990)), or confer a particular property on the target cell (eg. if the target cell was a tumour cell and the new sequence directed the expression of a tumour suppressing gene), or express an antisense construct designed to turn off a gene or set of genes in the target cell, or express a gene or gene product designed to be toxic to the target cell.

This technique of "targeted gene transfer" has a number of uses in research and also in therapy and diagnostics. For example, gene therapy often aims to target the replacement gene to a specific cell type that is deficient in its activity; targeted pAbs provide an answer to this problem. In diagnostics, phage specific for particular bacteria or groups of bacteria have been used to target marker genes, eg. luciferase, to the bacterial host (see, for example, Ulitzer, S., and Kuhn, J., EPA 85303913.9). If the host range of the phage is appropriate, only those bacteria that are being tested for, will be infected by the phage, express the luciferase gene and be detected by the light they emit. This system has been used to detect the presence of Salmonella. One major problem with this approach is the initial isolation of a bacteriophage with the correct host range and then cloning a luciferase gene cassette into that phage, such that it is functional. The pAb system allows the luciferase cassette to be cloned into a well characterised system (filamentous phage) and allows simple selection of an appropriate host range, by modifying the antibody (or other binding molecule) specificity the pAb contains.

The applicant has also shown that enzymes can be expressed on the phage surface. Useful applications of this invention include the cloning of enzyme coding genes, or the design and selection of mutant enzymes with enhanced properties on particular substrates. For example, conditions can be used whereby the enzyme (or modified enzyme) binds a particular substrate, product or

intermediate (or analogues of them) to identify phage from a library containing a desired activity or by subjecting phage already expressing the enzyme, to in vitro mutagenesis, followed by selection of those variants with a desired level of binding and/or catalysis.

The present applicants have also been able to develop novel screening systems and assay formats which depend on the unique properties of these packages eg phage antibodies.

The present invention provides a method for producing a package which method comprises the steps of:

- a) inserting a nucleotide sequence encoding the binding molecule within a viral genome;
- b) culturing the virus containing said nucleotide sequence, so that said binding molecule is expressed by the virus presented at its surface.

The present invention also provides a method for producing a binding molecule specific for a particular epitope which comprises producing a package as described above and the additional step of screening for said binding molecule by binding of said molecule to said epitope. The method may comprise one or more of the additional steps of: i) separating the package from the epitope; ii) recovering said package; and iii) using the inserted nucleotide sequence in a recombinant system to produce the binding molecule separate from virus. The screening step may isolate the nucleotide sequence encoding the binding molecule of desired specificity, by virtue of said binding molecule being expressed in association with the surface of the virus.

In the above methods, the binding molecule may be an antibody, or a fragment or derivative of an antibody. Alternatively, the binding molecule may be an enzyme or receptor and fragments/derivatives of any such enzymes or receptors.

In the above methods, the virus may be a filamentous F-specific bacteriophage. The filamentous F-specific bacteriophage may be fd. In particular, it may be a

tetracycline resistant version of fd known as fd-tet. The nucleotide sequence may be inserted within the gene III region of fd. The sequence may be inserted after the signal sequence of gene III, preferably after amino acid+1 of the mature protein. The site for insertion may be flanked by short sequences corresponding to sequences which occur at each end of the DNA to be inserted. For example, the protein domain is an immunoglobulin domain, the insertion site in the phage may be flanked by nucleotide sequences which code for the first five amino acids and the last five amino acids of the Ig domain. Such flanking nucleotide sequences are shown in figure 4(2) B and C, wherein the site-flanking nucleotide sequences encode amino acid sequences QVQLQ and VTVSS which occur at either end of the V_H domain, or QVQLQ and LEIKR which occur at either end of the F_V (combined V_H + V_L) domain. Each of these sequences flanking the insertion site may include a suitable cleavage site, as shown in Fig 4.

Alternatively, the flanking nucleotide sequences shown in figure 4(2) B and C as described above, may be used to flank the insertion site for any DNA to be inserted, whether or not that DNA codes an immunoglobulin.

In the above methods the nucleotide sequences inserted within the viral genome may be derived from eg mammalian spleen cells or peripheral blood lymphocytes. The mammal may be immunised or non-immunised. Alternatively, the nucleotide sequence may be derived by the in vitro mutagenesis of an existing antibody coding sequence. The phage particle presenting said binding molecule may remain intact and infectious.

As previously mentioned, the present invention also provides novel screening systems and assay formats. In these systems and formats the gene sequence encoding the binding molecule (eg the antibody) of desired specificity is separated from the general population having a range of specificities by the fact of its binding to a specific target (eg the antigen or epitope).

Thus, the present invention provides a method of screening a population of phage antibodies (where the binding molecule is an antibody) for a phage antibody with a desired specificity, which comprises contacting said population of phage antibodies with a desired epitope and separating phage antibody which binds to said epitope, from said epitope. The means for separating any binding phage antibodies may be varied in order to obtain binding phage antibodies with different binding affinities for said epitope.

Alternatively, in order to obtain high affinity phage antibodies the epitope may be presented to the population of phage antibodies already with a binding member for said epitope bound thereto, in which case, phage antibodies with a higher binding affinity for said epitope than said bound binding member will displace said bound binding member. The high affinity phage antibodies can then be separated from said epitope.

Separation of phage antibodies from said epitope may be achieved by eg elution techniques well known in the art, infection of suitable bacteria etc.

The present invention also provides packages as defined above and binding molecules (eg antibodies, enzymes, receptors; fragments and derivatives thereof), obtainable by use of any of the above defined methods, systems and formats.

The applicants have chosen the filamentous F- specific bacteriophages as an example of the type of phage that could provide a vehicle for the expression of antibodies and antibody fragments and derivatives on their surface and facilitate subsequent screening and manipulation.

The F-specific phages (eg f1, fd and M13) have evolved a method of propagation which does not kill the host cell and they are used commonly as vehicles for recombinant DNA (Kornberg, A., DNA Replication, W.H. Freeman and Co., San Francisco, 1980). The single stranded DNA genome (approximately 6.4 Kb) is extruded through the bacterial

membrane where it sequesters capsid sub-units, to produce mature virions. These virions are 6 nm in diameter, 1µm in length and each contain approximately 2,800 molecules of the major coat protein encoded by viral gene VIII and four
 5 molecules of the adsorption molecule encoded by viral gene III. The latter is located at one end of the virion. The structure has been reviewed by Webster et al., 1978 in *The Single Stranded DNA Phages*, 557-569, Cold Spring Harbor Laboratory Press. The gene III product is involved in the
 10 binding of the phage to the bacterial F-pilus.

Although these phages do not kill their host during normal replication, disruption of some of their genes can lead to cell death (Kornberg, A., 1980 supra.) This places some restraint on their use. The applicants have recognized
 15 that gene III of phage fd is an attractive possibility for the insertion of biologically active foreign sequences. The protein itself is only a minor component of the phage coat and disruption of the gene does not lead to cell death (Smith, G. 1988, *Virology* 167: 156-165). Furthermore, it is
 20 possible to insert some foreign sequences (with no biological function) into various positions within this gene (Smith, G., 1985 *Science* 228: 1315-1317., Parmley, S.F. and Smith, G.P *Gene*: 73 (1988) p. 305-318., and de la Cruz, V.F., et al., 1988, *J. Biol. Chem.*, 263: 4318-4322). In
 25 these cases, although the infectivity of the virion was disrupted, the inserted sequences could be detected on the phage surface.

The protein encoded by gene III has several domains (Pratt, D., et al., 1969 *Virology* 39:42-53., Grant, R.A., et
 30 al., 1981, *J. Biol. Chem.* 256: 539-546 and Armstrong, J., et al., *FEBS Lett.* 135: 167-172 1981.) including: i) a signal sequence that directs the protein to the cell membrane and which is then cleaved off; ii) a domain that anchors the mature protein into the bacterial cell membrane (and also
 35 the phage coat); and iii) a domain that specifically binds to the phage receptor the F-pilus of the host bacterium. Short sequences derived from protein molecules have been

inserted into two places within the mature molecule (Smith, G., 1985 supra., and Parmley, S.F. and Smith, G.P., 1988 supra.) into an inter-domain region and also between amino acids 2 and 3 at the N-terminus. The insertion sites at the N-terminus were more successful in maintaining the structural integrity of the gene III protein and displaying the peptides on the surface of the phage. By use of specific antisera, the peptides inserted into this position were shown to be on the surface of the phage. These authors were also able to purify the phage using this property. However, the peptides expressed by the phage, did not possess measurable biological functions of their own.

Retaining the biological function of a molecule when it is expressed in a radically different context to its natural state is difficult. The demands on the structure of the molecule are heavy. In contrast, retaining the ability to be bound by specific antisera is a passive process which imposes far less rigorous demands on the structure of the molecule. For example, it is the rule rather than the exception that polyclonal antisera will recognise totally denatured, and biologically inactive, proteins on Western blots (see for example, Harlow, E. and Lane, D., Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory Press 1988). Therefore, the insertion of peptides into a region that allows their structure to be probed with antisera teaches only that the region allows the inserted sequences to be exposed and does not teach that the region is suitable for the insertion of large sequences with demanding biological function.

This experience with Western blots is a graphic practical demonstration which shows that retaining the ability to be bound by specific antisera imposes far less rigorous demands on the structure of a molecule, than does the retention of a biological function.

The applicants have investigated the possibility of inserting biologically active antibody fragments into the gene III region of fd to create a large fusion protein. As

is apparent from the previous discussion, this approach makes onerous demands on the functionality of the fusion protein. The insertion is large, 100-200 amino acids; the antibody derived domain must fold efficiently and correctly to retain antigen-binding; and most of the functions of gene III must be retained. The applicants approach to the construction of the fusion molecule was designed to minimise the risk of disrupting these functions. The initial vector used was fd-tet (Zacher, A.N., et al., 1980, Gene 9, 127-140) a tetracycline resistant version of fd bacteriophage that can be propagated as a plasmid that confers tetracycline resistance to the infected E.coli host. The applicants chose to insert after the signal sequence of the fd gene III protein for several reasons. In particular, the applicants chose to insert after amino acid 1 of the mature protein to retain the context for the signal peptidase cleavage. To retain the structure and function of gene III itself, the majority of the original amino acids are synthesized after the inserted immunoglobulin sequences. The inserted immunoglobulin sequences were designed to include residues from the switch region that links V_H - V_L to C_H1 - C_L (Lesk, A., and Chothia, C., Nature 335, 188-190, 1988).

Surprisingly, by manipulating gene III of bacteriophage fd, the present applicants have been able to construct a bacteriophage that expresses on its surface large biologically functional antibody molecules and which remains intact and infectious. Furthermore, the phages bearing antibodies of the correct specificity, can be selected from a background where the majority of phages do not show this specificity.

The population of antibody molecules inserted into the phage can be derived from a variety of sources. For example, immunised or non-immunised rodents or humans, and from organs such as spleen and peripheral blood lymphocytes. The coding sequences are derived from these sources by techniques familiar to those skilled in the art (Orlandi,

R., et al., 1989 supra; Larrick, J.W., et al., 1989 supra; Chiang, Y.L., et al., 1989 Bio Techniques 7, p. 360-366; Ward, E.S, et al., 1989 supra; Sastry, L., et al., 1989 supra.) Each individual phage antibody in the
5 resulting library of phage antibodies will express antibody derived fragments that are monoclonal with respect to its antigen-binding characteristics.

The disclosure made by the present applicants is important and provides a significant breakthrough in the
10 technology relating to the production of biological binding molecules, their fragments and derivatives by the use of recombinant methods.

In standard recombinant techniques for the production of antibodies, an expression vector containing sequences
15 coding for the antibody polypeptide chains is used to transform eg E.coli. The antibody polypeptides are expressed and detected by use of standard screening systems. When the screen detects an antibody polypeptide of the desired specificity, one has to return to the particular
20 transformed E.coli expressing the desired antibody polypeptide. Furthermore, the vector containing the coding sequence for the desired antibody polypeptide then has to be isolated for use from E.coli in the further processing steps.

25 In the present invention however, the desired antibody polypeptide when expressed, is already packaged with its gene coding sequence. This means that when the screen detects an antibody polypeptide of desired specificity, there is no need to return to the original culture for
30 isolation of that sequence.

Because the phage antibody is a novel structure that contains an antibody of monoclonal antigen-binding specificity on the surface of a relatively simple structure also containing the genetic information encoding its
35 function, phage antibodies that bind antigen can be recovered very efficiently by either eluting off (eg using diethylamine, high salt etc) and infecting suitable

bacterial or by denaturing the structure and specifically amplifying the antibody encoding sequences using PCR. That is, there is no necessity to refer back to the original bacterial clone that gave rise to the phage antibody.

5 Individual phage antibodies expressing the desired antigen-binding specificity can be isolated from the complex library using the conventional screening techniques (eg as described in Harlow, E., and Lane, D., 1988, supra). One example is illustrated in figure 2(i). This shows antigen
10 (ag) bound to a solid surface (s). The population of phage antibodies is then passed over the antigen, and those individuals p that bind are retained after washing, and optionally detected with detection system d. One possible
15 detection system based upon anti-fd antisera is illustrated below in example 4. Since the bound phage antibody can be amplified using for example PCR or bacterial infection, it is also possible to rescue the desired specificity even when insufficient individuals are bound to allow detection via conventional techniques.

20 The efficiency of this screening procedure for phage antibodies and the ability to create very large libraries means that the immunisation techniques developed to increase the proportion of screened cells producing antibodies of interest will not be an absolute requirement. The technique
25 allows the rapid isolation of antigen-binding specificities, including those that would be difficult or even unobtainable by conventional techniques, for example, catalytic or anti-idiotypic antibodies. Removal of the animal altogether is now possible once a complete library of the immune
30 repertoire has been constructed.

Affinity Maturation Screening Formats

The applicants have also devised a series of novel screening techniques that are practicable only because of the unique properties of phage antibodies. The general
35 outline of some screening procedures is illustrated in figure 2.

The population/library of phage antibodies to be

screened could be generated from immunised or other animals; or be created in vitro by mutagenising pre-existing phageantibodies (using techniques well-known in the art such as oligonucleotide directed mutagenesis (Sambrook, J., et al., 1989 Molecular Cloning a Laboratory Manual, Cold Spring Harbor Laboratory Press). This population can be screened in one or more of the formats described below with reference to figure 2, to derive those individual phage antibodies whose antigen binding properties are different from sample c. Examples of the possible screening formats are:

Binding/Elution

Referring to figure 2(i) population p binds to antigen ag fixed to a solid support s. If samples of bound population p are removed under increasingly stringent conditions, the binding affinity represented in each sample will increase. Conditions of increased stringency can be obtained, for example, by increasing the time of soaking or changing the pH of the soak solution, etc.

Competition

Referring to figure 2(ii) antigen ag can be bound to a solid support s and bound to saturation by the original binding molecule c. If a population of mutant phage antibody (or a set of unrelated phage antibody) p is offered to the complex, only those that have higher affinity for antigen ag than c will bind. In most examples, only a minority of population c will be displaced by individuals from population p. If c is a traditional antibody molecule, all bound material can be recovered and bound p recovered by infecting suitable bacteria and/or by use of standard techniques such as PCR.

An advantageous application is where ag is used as a receptor and c the corresponding ligand. The recovered bound population p is then related structurally to the receptor binding site/and or ligand. This type of specificity is known to be very useful in the pharmaceutical

industry.

Another advantageous application is where a_g is an antibody and c its antigen. The recovered bound population p is then an anti-idiotypic antibody which have numerous uses in research and the diagnostic and pharmaceutical industries.

In some instances it may prove advantageous to pre-select population p . For example, in the anti-idiotypic example above, p can be absorbed against a related antibody that does not bind the antigen.

However, if c is a phage antibody, then either or both c and p can advantageously be marked in some way to both distinguish and select for bound p over bound c . This marking can be physical, for example, by pre-labelling p with biotin; or more advantageously, genetic. For example, c can be marked with an EcoB restriction site, whilst p can be marked with an EcoK restriction site (see Carter, P. et al., 1985, Nucl. Acids Res. 13, 4431-4443). When bound $p+c$ are eluted from the antigen and used to infect bacteria, there is restriction (and thus no growth) of population c (i.e. EcoB restricting bacteria in this example). Any phage that grew, would be greatly enriched for those individuals from p with higher binding affinities. Alternatively, the genetic marking can be achieved by marking p with new sequences, which can be used to specifically amplify p from the mixture using PCR.

The novel structure of the phage antibody molecule can be used in a number of other applications some examples of which are:

Signal Amplification

Acting as a novel molecular entity in itself, phage antibodies combine the ability to bind the specific antigen with the amplification, if the major coat protein is used to attach another moiety. This moiety can be attached via immunological, chemical, or any other means and can be used,

for example, to label the complex with detection reagents or cytotoxic molecules for use in vivo or in vitro.

Physical Detection

The size of the phage antibody can be used as a marker particularly with respect to physical methods of detection such as electron microscopy and/or some biosensors, eg. surface plasmon resonance.

Diagnostic Assays

The phage antibody molecule also has advantageous uses in diagnostic assays, particularly where separation can be effected using its physical properties for example centrifugation, filtration etc.

In order that the invention is more fully understood, embodiments will now be described in more detail by way of example only and not by way of limitation with reference to the figures described below.

Figure 1 shows the basic structure of the simplest antibody molecule IgG.

Figure 2 shows schematically novel screening techniques which utilise the unique properties of phage antibodies.

Figure 3 shows a scheme for the construction of vectors.

Figure 4 shows the nucleotide sequences for the oligonucleotides and vectors. All sequences are drawn 5' to 3' and are numbered according to Beck et al., 1978, Nucl. Acid Res., 5: 4495-4503. 4.1 shows the sequences of the oligonucleotides used for mutagenesis (oligo's 1 and 2) or sequencing (oligo 3). The sequences shown were synthesized on an Applied Biosystems, oligonucleotide synthesizer and are complementary to the single stranded form of fd-tet (they are in the anti-sense form with respect to gene III). 4.2 shows the sequences of the various constructs around the gene III insertion site. these sequences are drawn in the sense orientation with respect to gene III; a) fd-tet (and

FDTdBst) b) FDTPs/Bs and c) FDTPs/Xh. The key restriction enzyme sites are shown along with the immunoglobulin amino acids contributed by the vectors, (amino acid single letter code is used, see Harlow, E., and Lane, D., 1988 supra.).

Figure 5 shows the nucleotide and amino acid sequences for scFv in the vector scFvD1.3 myc. This gives the sequence of the anti-lysozyme single chain Fv and surrounding sequences in scFvD1.3 myc showing the N-terminal pel B signal peptide sequence and the C-terminal myc tag sequence (Ward, E.S., et al., 1989, supra.). Also highlighted is the peptide sequence linking the V_H and V_L regions. The amino acid sequence is represented above the nucleotide sequence by the single letter code, see Harlow, E., and Lane, D., 1988 supra..

Figure 6 shows the effect of varying the amount of supernatant on the binding of phage antibodies to lysozyme in graphical form. Each point is the average of duplicate samples. Lysozyme was coated at 1 mg/ml in 50 mM NaHCO₃.

Figure 7 shows the effect of varying the coating concentration on the binding of phage antibodies to lysozyme in graphical form. Each point is the average of duplicate samples. Lysozyme was coated with the specified concentration of either BSA or lysozyme.

Figure 8 shows the sequence around the cloning site in gene III of fd-Cat2.

Figure 9 shows the binding of pAb (D1.3) to lysozymes. Binding of phage as detected by ELISA to (a) hen egg-white lysozyme (HEL) (b) turkey egg-white lysozyme (TEL), (c) human lysozyme (HUL), (d) bovine serum albumin (BSA). A further control of (c) fd-CAT1 to HEL.

Figure 10 shows a map of fab D1.3 in pUC19.

Figure 11 shows the ELISA results providing a comparison of lysozyme-binding by phage-Fab and phage-ScFv. Vector=fd-CAT2 (example 5); fdSCFV(OX)=pAbNQ11 (Example 9); fdVHCH1(D1.3)=grown in normal cells (ie. no light chain, see

example 7); fdfab-fdfab-fdVHCH1 (D1.3) grown in cells containing D1.3 light chain; fdSCFV (D1.3)=pAbD1.3.

Figure 12 shows oligonucleotide probing of affinity purified phage. 10^{12} phage in the ratio 1 pAb (D1.3) in
5 4×10^4 fd-CAT1 phages were affinity purified and probed with an oligonucleotide specific for pAb (D1.3) (a) filter after one round of affinity purification (900 colonies total) and, (b) after two rounds (372 colonies total).

10 Figure 13 shows the sequence of the anti-oxazalone antibody NQ11.

Figure 14 shows the ELISA results for binding of pAb NQ11 and pAb D1.3 and Vector FDTPs/xh to specified antigens.

15 Figure 15 shows the sequence surrounding phoA insertion in fd-phoA1. The restriction sites used for cloning are shown, as well as the amino acids encoded by phoA around the insertion site. The first five amino acids of the mature fusion come from gene 3.

20 Figure 16(1) shows the structure of gene 3 and figure 16(2) shows the peptide linker sites A and B.

Figure 17 shows schematically the protocol for PCR assembly of mouse VH and VLK repertoires for phage display described in example 16.

25 Figure 18 shows examples of the final products obtained with the procedure of example 16. Lanes a and b

show the products of the initial PCR using heavy and light chain primers respectively; lane c shows the complete assembled 700bp product before final digestion with NotI and ApalI; M1, M2 markers Φ 174 Hae III digest
5 and 123 base pair ladder (BRL Limited, P.O. Box 35, Washington Road, Paisley, Scotland) respectively.

Figure 19 shows the binding of ^{125}I -PDGF-BB to fd h-PDGFB-R phage in immunoprecipitation assay and comparison to fd TPs/Bs and no phage controls; binding is expressed
10 as a percentage of the total ^{125}I -PDGF-BB added to the incubation.

Figure 20 shows the displacement of ^{125}I -PDGF-BB bound to fd-h-PDGFB-R phage using unlabelled PDGF-BB measured using an immunoprecipitation assay. Binding is
15 expressed as a percentage of the total ^{125}I -PDGF-BB added to the incubation.

Figure 21 shows the displacement of ^{125}I -PDGF-BB bound to fd-h-PDGFB-R phage using unlabelled PDGF-BB measured using an immunoprecipitation assay. Non-
20 specific binding of ^{125}I -PDGF-BB to vector phage fdTPs/Bs in the absence of added unlabelled PDGF was deducted from each point.

Figure 22 shows the results of an ELISA of lysozyme binding by pCAT-3 ScFv D1.3 phagemid in comparison with
25 pCAT-3 vector (both rescued by M13K07) and fdCAT2 ScFv D1.3 as described in example 17. The ELISA was performed

as described in example 6 with modifications detailed in example 17.

The following procedures used by the present applicants are described in Sambrook, J. et al., 1989, supra.: restriction digestion, ligation, preparation of competent cells (Hanahan method), transformation, analysis of restriction enzyme digestion products on agarose gels, purification of DNA using phenol/chloroform, 5'-end labelling of oligonucleotides, filter screening of bacterial colonies, preparation of 2xTY medium and plates, preparation of tetracycline stock solutions, PAGE of proteins, preparation of phosphate buffered saline.

All enzymes were supplied by New England Biolabs (CP Laboratories, PO Box 22, Bishop's Stortford, Herts., England) and were used according to manufacturer's instructions unless otherwise stated.

The vector fd-tet (Zacher, A.N. et al., 1980, supra) was obtained from the American Type Culture Collection (ATCC

No. 37000) and transformed into competent TG1 cells (genotype: K12 δ (lac-pro), sup E, thi, hsdD5/F'traD36, pro A+B+, Lac I^q, lac δ M15).

Viral particles were prepared by growing TG1 cells containing the desired construct in 10 to 100 mls 2xTY medium with 15 μ g/ml tetracycline for 16-24 hours. The culture supernatant was collected by centrifugation for 10 mins at 10,000 rpm in an 8 x 50 ml rotor, Sorval RC-5B centrifuge. Phage particles were precipitated by adding 1/5th volume 20% polyethylene glycol (PEG)/2.5M NaCl and leaving at 4°C for 1 hour. These were spun for 15 minutes as described above and the pellets resuspended in 10 mM Tris/HCl pH 8, 1 mM EDTA to 1/100th of the original volume. Residual bacteria and undissolved material were removed by spinning for 2 minutes in a microcentrifuge. Single stranded DNA for mutagenesis or sequencing was prepared from concentrated phage according to Sambrook, J., et al., 1989, *supra*.

Example 1

Design of Insertion Point Linkers and Construction of Vectors

The vector fd-tet has two BstEII restriction sites flanking the tetracycline resistance gene (fig 3). Since the strategy for inserting the V_H fragments was to ligate them into a newly inserted BstEII site within gene III, it was advantageous to delete the original BstEII sites from fd-tet. This was achieved by digesting fd-tet with the restriction enzyme BstEII, filling-in the 5' overhangs and re-ligating to generate the vector FDT6Bst. Digestion of fd-tet with BstEII (0.5 units/ μ l) was carried out in 1x KGB buffer (100 mM potassium glutamate, 25 mM Tris-acetate (PH 7.5), 10 mM magnesium acetate, 50 μ g/ml bovine serum albumin, 0.5 mM (Sambrook, J., et al., 1989, *supra*.) with DNA at a concentration of 25 ng/ μ l. The 5' overhang was filled in, using 2x KGB buffer, 250 μ M each dNTP's

(Pharmacia Ltd., Pharmacia House, Midsummer Boulevard, Milton Keynes, Bucks., UK.) and Klenow Fragment (Amersham International, Lincoln Place, Green End, Aylesbury, Bucks., UK) at 0.04 units/ μ l. After incubating for 1 hour at room temperature, DNA was extracted with phenol/chloroform and precipitated with ethanol.

Ligations were carried out at a DNA concentration of 50ng/ μ l for 1 hour at room temperature using T4 DNA ligase (40 units/ μ l). Ligations were transformed into competent TG1 cells and plated onto TY plates supplemented with 15 μ g/ml tetracycline. Colonies were picked into 25 mls of 2xTY medium supplemented with 15 μ g/ml tetracycline and grown overnight at 37°C.

Double stranded DNA was purified from the resulting clones using the gene-clean II kit (Biol01 Inc., PO Box 2284, La Jolla, California, 92038-2284, USA.) and according to the small scale rapid plasmid DNA isolation procedure described therein. The orientation of 5 of the resulting clones was checked using the restriction enzyme Clal. A clone was chosen which gave the same Clal pattern as fd-tet, but which had no BstE II sites.

In vitro mutagenesis of FDT6Bst was used to generate vectors that facilitated cloning of antibody fragments downstream of the gene III signal peptide and in frame with the gene III coding sequence. The oligonucleotide directed mutagenesis system, version 2 (Amersham International) was used with oligo 1 (figure 4) to create FDTPs/Bs. The sequence of FDTPs/Bs (figure 4) was confirmed using the sequenase version 2.0 kit (USB Corp., PO Box 22400, Cleveland, Ohio, 44122, USA.) with oligo 3 (figure 4) as a primer.

A second vector FDTPs/Xh (to facilitate cloning of single chain F_v fragments) was generated by mutagenising FDTPs/Bs with oligo 2 according to the method of Venkitaraman, A.R., Nucl. Acid Res. 17, p 3314. The

sequence of FDTPs/Xh (figure 4) was confirmed using the sequenase version 2.0 kit (USB Corp.) with oligo 3 as a primer.

Clearly, alternative constructions will be apparent to those skilled in the art. For example, M13 and/or its host bacteria could be modified such that its gene III could be disrupted without the onset of excessive cell death; the modified fd gene III, or other modified protein, could be incorporated into a plasmid containing a single stranded phage replication origin, such as pUC119, superinfection with modified phage such as K07 would then result in the encapsulation of the phage antibody genome in a coat partially derived from the helper phage and partly from the phage antibody gene III construct.

The detailed construction of a vector such as FDTPs/Bs is only one way of achieving the end of a phage antibody. For example, techniques such as sticky feet cloning/mutagenesis (Clackson, T. and Winter, G. 1989 Nucl. Acids. Res., 17, p 10163-10170) could be used to avoid use of restriction enzyme digests and/or ligation steps.

Example 2.

Insertion of Immunoglobulin F_V Domain into Phage Antibody

The plasmid scFv D1.3 myc (gift from G. Winter and A. Griffiths) contains V_H and V_L sequences from the antibody D1.3 fused via a peptide linker sequence to form a single chain F_V version of antibody D1.3. The sequence of the scF_V and surrounding sequences in scFvD1.3 myc is shown in figure 5.

The D1.3 antibody is directed against hen egg lysozyme (Harper, M. et al., 1987, Molec. Immunol. 24, 97-108) and the scFv form expressed in E. coli has the same specificity (A. Griffiths and G. Winter personal Communication).

Digestion of scFv D1.3 myc with PstI and XhoI, excises a fragment of 693 bp which encodes the bulk of the scFv. Ligation of this fragment into FDTPs/Xh cleaved with PstI

and XhoI gave rise to the construct FDTSCFVD1.3 encoding the gene III signal peptide and first amino acid fused to the complete D1.3 scFv, followed by the mature gene III protein from amino acid 2.

The vector FDTPs/Xh was prepared for ligation by digesting with the PstI and XhoI for 2 hours followed by digestion with calf intestinal alkaline phosphatase (Boehringer Mannheim UK Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG) at one unit/ul for 30 minutes at 37°C. Fresh calf intestinal alkaline phosphatase was added to a final total concentration of 2 units/ul and incubated for a further 30 minutes at 37°C. The reaction was extracted three times with phenol/chloroform, precipitated with ethanol and dissolved in water. The insert from scFvD1.3 myc was excised with the appropriate restriction enzymes, extracted twice with phenol/chloroform, precipitated with ethanol and dissolved in water. Ligations were carried out as described in example 1 except both vector and insert samples were at a final concentration of 5 ng/ul each. The formation of the correct construct was confirmed by sequencing as described in example 1.

To demonstrate that proteins of the expected size were produced, virions were concentrated by PEG precipitation as described above, and the equivalent of 2mls of supernatant was loaded onto an 18% SDS polyacrylamide gel. After electrophoresis, the gel was soaked in gel running buffer (50 mM Tris, 380 mM Glycine, 0.1%SDS) with 20% methanol for 15 minutes. Transfer to nitrocellulose filter was executed in fresh 1x running buffer/20% methanol using TE70 Semi Phor a semi-dry blotting apparatus (Hoeffer, 654 Minnesota Street, Box 77387, San Francisco, California 94107, USA.).

After transfer, the filter was blocked by incubation for 1 hour in a 2% solution of milk powder (Cadbury's Marvel) in phosphate buffered saline (PBS). Detection of F_y and V_H protein sequences in the phage antibody fusion proteins was effected by soaking the filter for 1 hour with a 1/1000 dilution (in 2% milk powder) of a rabbit polyclonal

antiserum raised against affinity purified, bacterially expressed F_V fragment (gift from G. Winter). After washing PBS (3 x 5 minute washes), bound primary antibody was detected using an anti-rabbit antibody conjugated to horseradish peroxidase (Sigma, Fancy Road, Poole, Dorset, BH17 7NH, UK.) for 1 hour. The filter was washed in PBS/0.1% triton X-100 and developed with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.02% cobalt chloride, 0.03% hydrogen peroxide in PBS.

The results show that with FDTVHD1.3 (from example 3) and FDTSCVFVD1.3, a protein of between 69,000 and 92,500 daltons is detected by the anti-F_V serum. This is the expected size for the fusion proteins constructed. This product is not observed in supernatants derived from fd-tet, FDTδBst or FDTPs/Xh.

Example 3.

Insertion of Immunoglobulin V_H Domain into Phage Antibody

The V_H fragment from D1.3 was generated from the plasmid pSW1-VHD1.3-TAG1 (Ward, E.S. et al., 1989 supra.). Digestion with PstI and BstEII generates the fragment shown between positions 113 and 432 in figure 5. Cloning of this fragment into the PstI and BstEII sites of FDTPs/Bs gave rise to the construct FDTVHD1.3 which encodes a fusion protein with a complete V_H inserted between the first and third amino acids of the mature gene III protein (amino acid two has been deleted).

The methods used were exactly as in example 2 except that the vector used was FDTPs/Bs digested with PstI and BstEII.

Example 4.

Analysis of Binding Specificity of Phage Antibodies

The binding of the various phage antibodies to the specific antigen, lysozyme, was analysed using ELISA techniques. Phage antibodies were grown in E.coli and phage antibody particles were precipitated with PEG as in the materials and methods. Bound phage antibody particles were detected using polyclonal rabbit serum raised against the

closely related phage M13.

ELISA plates were prepared by coating 96 well plates (Falcon Microtest III flexible plate. Falcon: Becton Dickinson Labware, 1950 Williams Drive, Oxnard, California, 93030, USA.) with 200 μ l of a solution of lysozyme (1mg/ml unless otherwise stated) in 50 mM NaHCO_3 for 16-24 hours. Before use, this solution was removed, the plate rinsed several times in PBS and incubated with 200 μ l of 2% milk powder/PBS for 1 hour. After rinsing several times with PBS, 100 μ l of the test samples were added and incubated for 1 hour. Plates were washed (3 rinses in 0.05% Tween 20/PBS followed by 3 rinses in PBS alone). Bound phage antibodies were detected by adding 200 μ l/well of a 1/1000 dilution of sheep anti-M13 polyclonal antiserum (gift from G. Winter) in 2% milk powder/PBS and incubating for 1 hour. After washing as above, plates were incubated with biotinylated anti-sheep antibody (Amersham International) for 30 minutes. Plates were washed as above, and incubated with streptavidin-horseradish peroxidase complex (Amersham International). After a final wash as above, 0.5 mg/ml ABTS substrate in citrate buffer was added (ABTS=2'2'-azinobis(3-ethylbenzthiazoline sulphonate); citrate buffer =50 mM citric acid, 50 mM tri-sodium citrate at a ratio of 54:46). Hydrogen peroxide was added to a final concentration of 0.003% and the plates incubated for 1 hour. The optical density at 405 nm was read in a Titertek multiskan plate reader.

Figure 6 shows the effect of varying the amount of phage antibody. 100 μ l of various dilutions of PEG precipitated phage were applied and the amount expressed in terms of the original culture volume from which it was derived. Signals derived from both the scFv containing phage antibody (FDTSCFVD1.3) and the V_H containing phage antibody (FDTVHD1.3) were higher than that derived from the phage antibody vector (FDTPs/Xh). The highest signal to noise ratio occurs using the equivalent of 1.3 mls of culture.

Figure 7 shows the results of coating the plates with varying concentrations of lysozyme or bovine serum albumin (BSA). The equivalent of 1 ml of the original phage antibody culture supernatant was used. The signals from supernatants derived from FDTSCFVD1.3 were again higher than those derived from FDTPs/Xh when lysozyme coated wells were used. There was no significant difference between these two types of supernatant when the plates were coated with BSA. Broadly speaking the level of signal on the plates is proportional to the amount of lysozyme coated. These results demonstrate that the binding detected is specific for lysozyme as the antigen.

Example 5.

Construction of fd Cat 2

It would be useful to design vectors that enable the use of restriction enzymes that cut DNA infrequently, thus avoiding unwanted digestion of the antibody gene inserts within their coding sequence. Enzymes with an eight base recognition sequence are particularly useful in this respect, for example NotI and SfiI. Chaudhary et al (PNAS 87 p1066-1070, 1990) have identified a number of restriction sites which occur rarely in antibody variable genes. The applicant has designed and constructed a vector that utilises two of these sites, as an example of how this type of enzyme can be used. Essentially sites for the enzymes ApaI and NotI were engineered into FDTPs/Xh to create fdCAT2.

The oligonucleotide:

5'ACT TTC AAC AGT TTC TGC GGC CGC CCG TTT GAT CTC GAG CTC
CTG CAG TTG GAC CTG TGC ACT GTG AGA ATA GAA 3'

was synthesised (supra fig 4 legend) and used to mutagenise FDTPs/Xh using an in vitro mutagenesis kit from Amersham International as described in example 1, to create fd-CAT2. The sequence of fd-CAT2 was checked around the site of manipulation by DNA sequencing. The final sequence around the insertion point within gene III is shown in figure 8.

Example 6

Specific Binding of Phage-antibody(pAb) to Antigen

The binding of pAb D1.3 (FDTSCFVD1.3 of example 2) to lysozyme was further analysed by ELISA.

Methods.

1. Phage growth.

Cultures of phage transduced bacteria were prepared in 10-100 mls 2 x YT medium with 15 µg/ml tetracycline and grown with shaking at 37°C for 16-24 hrs. Phage supernatant was prepared by centrifugation of the culture (10 min at 10,000 rpm, 8 x 50 ml rotor, Sorval RC-5B centrifuge). At this stage, the phage titre was $1 - 5 \times 10^{10}$ ml⁻¹ transducing units. The phage were precipitated by adding 1/5 volume 20% polyethylene glycol, 2.5 M NaCl, leaving for 1 hr at 4°C, and centrifuging (supra). The phage pellets were resuspended in 10 mM Tris-HCl, 1mM EDTA pH 8.0 to 1/100th of the original volume, and residual bacteria and aggregated phage removed by centrifugation for 2 min in a bench microcentrifuge.

ELISA

Plates were coated with antigen (1 mg ml⁻¹ antigen) and blocked as described in example 4. 2×10^{10} phage transducing units were added to the antigen coated plates in phosphate buffered saline (PBS) containing 2% skimmed milk powder (MPBS). Plates were washed between each step with three rinses of 0.5% Tween-20 in PBS followed by three rinses of PBS. Bound phage was developed by incubating with sheep anti-M13 antisera and detected with horseradish peroxidase (HRP) conjugated anti-goat serum (Sigma, Poole, Dorset, UK) and ABTS (2'2'-azinobis (3-ethylbenzthiazoline sulphonic acid)). Readings were taken at 405 nm after a suitable period. The results (figure 9) show that the antibody bearing-phage had the same pattern of reactivity as the original D1.3 antibody (Harper, M., Lema, F., Boulot, G., and Poljak, F.J. (1987) *Molec. Immunol.* 24, 97-108), and bound to hen egg-white lysozyme, but not to turkey egg-white lysozyme, human lysozyme or bovine serum albumin. The specificity of the phage is particularly illustrated by the

lack of binding to the turkey egg-white lysozyme that differs from hen egg-white lysozyme by only 7 amino acids.

Example 7

Expression of Fab D1.3 in fd CAT2

The aim of this example was to demonstrate that the scFv format used in example 2 was only one way of displaying antibody fragments in the pAb system. A more commonly used antibody fragment is the Fab fragment (figure 1) this example describes the construction of a pAb that expresses a Fab-like fragment on its surface and shows that it binds specifically to its antigen. The applicant chose to express the heavy chain of the antibody fragment consisting of the VH1 and CH1 domains in the pAb itself and to co-express the light chain in the same cell; the VH and CH1 regions of anti-lysozyme antibody D1.3 were cloned in fd CAT2, and the corresponding light chain cloned in plasmid pUC19. The light chain associates with the heavy chain-gene III fusion as it does when the heavy and light chains are expressed as independant proteins (Skerra, A and Pluckthun, A. Science 240, p1038-1040 (1988)).

It is possible to express the light chain from within the pAb genome by, for example, cloning an expression cassette into a suitable place in the phage genome. Such a suitable place would be the intergenic region which houses the multicloning sites engineered into derivative of the related phage M13 (see, for example, Yanisch-Perron, C. et al., Gene 33, p103-119, (1985)).

The starting point for this example was the clone Fab D1.3 in pUC19, a map of which is shown in figure 10. The sequence encoding the VH-CH1 region was PCR amplified from Fab D1.3 in pUC19 using oligonucleotides KSJ 6 and 7, which retain the Pst I site at the 5'end and introduce a Xho I site at the 3'end, to facilitate cloning into fd CAT2. The sequences for the oligonucleotides KSJ 6 and 7 are shown below.

KSJ6: 5' AGG TGC AGC TGC AGG AGT CAG G 3'

KSJ7: 5' GGT GAC CTC GAG TGA AGA TTT GGG CTC AAC TTT C 3'

PCR conditions were as described in example II, except that thirty cycles of PCR amplification were performed with denaturation at 92°C for 45 seconds, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. The template used was TG1 cells containing Fab D1.3 in pUC19 resuspended in water and boiled (see example 12). This regime resulted in amplification of the expected fragment of approximately 600bp. This fragment was cut with Pst I and Xho I, purified from an agarose gel and ligated into Pst I/Xho I-cut fd CAT2 as described in example 12. Part of the ligation was transformed into *E.coli* MC1061 (Available from, for example Clontech Laboratories Inc, Palo Alto, California) and colonies identified by hybridisation with the oligonucleotide D1.3CDR3A as described in example 10. The presence of the VHCH1 gene fragment was likewise confirmed by PCR, using oligonucleotides KSJ6 and 7. A representative clone was called fd CAT2: VHCH1 D1.3.

The heavy chain was deleted from fab D1.3 in pUC19 by Sph I cleavage of fab D1.3 plasmid DNA. The 2.7Kb fragment containing pUC19 and the light chain gene was purified from a TAE agarose gel, and 10ng of this DNA self-ligated and transformed into competent *E.coli* TG1. Cells were plated on 2YT agar containing ampicillin (100µg/ml) and incubated at 30°C overnight. The resulting colonies were used to make miniprep DNA (Sambrook et al. supra), and the absence of the heavy chain gene confirmed by digestion with Sph I and Hind III. A representative clone was called LC D1.3 DHC.

An overnight culture of fd CAT2; VHCH1 D1.3 cells was microcentrifuged at 13,000Xg for 10 minutes and 50µl of the supernatant containing phage particles added to 50µl of an overnight culture of LC D1.3 DHC cells. The cells were incubated at 37°C for 10 minutes and plated on 2YT agar containing ampicillin (100µg/ml) and 15mg/ml tetracycline. Phage were prepared from some of the resulting colonies and assayed for their ability to bind lysozyme as described in example 6.

The results (Figure 11) showed that when the heavy and

light chain Fab derivatives from the original antibody D1.3 were present, the pAb bound to lysozyme. pAb expressing the fd VHCH1 fragment did not bind to lysozyme unless grown in cells also expressing the light chain. This shows that a functional Fab fragment was produced by an association of the free light chain with the VHCH1 fragment fused to gene III and expressed on the surface of the pAb.

Example 8

Isolation of Specific, Desired Phage from a Mixture of Vector Phage.

The applicant purified pAb (D1.3) (originally called FDTSCFVD1.3 in example 2) from mixtures using antigen affinity columns. pAb (D1.3) was mixed with vector fd phage (see table 1) and approximately 10^{12} phage passed over a column of lysozyme-Sepharose (prepared from cyanogen bromide activated sepharose 4B (Pharmacia, Milton Keynes, Bucks, UK.) according to the manufacturers instructions. TG1 cells were infected with appropriate dilutions of the elutes and colonies derived, were analysed by probing with an oligonucleotide that detects only the pAb (D1.3) (see Table 1 and Fig. 12) A thousand fold enrichment of pAb (D1.3) was seen with a single column pass. By growing the enriched phage and passing it down the column again, enrichments of up to a million fold were seen.

Enrichment was also demonstrated using purely immunological criteria. For example, 10^{12} phage (at a ratio of 1 pAb (D1.3) to 4×10^6 FDTPs/Bs) was subjected to two rounds of affinity selection, and then 26 colonies picked and grown overnight. The phage was then assayed for lysozyme binding by ELISA (as example 6). Five colonies yielded phage with lysozyme binding activities, see table 1, and these were shown to encode the scFv (D1.3) by PCR screening (example 13, using 30 cycles of 1 minute at 92°C , 1 minute at 60°C , 1 minute at 72°C using CDR3PCR1 and oligo 3 (fig. 4) as primers).

Thus very rare pAbs can be fished out of large populations, by using antigen to select and then screen the phage.

Methods

Affinity Chromatography of pAbs

Approximately 10^{12} phage particles in 1ml MPBS were loaded onto a 1 ml lysozyme-Sepharose affinity column which had been prewashed in MPBS. The column was washed in turn with 10 ml PBS; then 10 ml 50 mM Tris-HCl, 500 mM NaCl pH 7.5; then 10ml 50 mM Tris-HCl, 500 mM NaCl pH 8.5; then 5 mls 50 mM Tris-HCl, 500 mM NaCl pH 9.5 (adjusted with triethylamine) and then eluted with 5 ml 100 mM triethylamine. The eluate was neutralised with 0.5 M sodium phosphate buffer pH 6.8 and the phage plated for analysis. For a second round of affinity chromatography, the first column eluate was plated to about 30,000 colonies per petri dish. After overnight growth, colonies were then scraped into 5 ml 2 x YT medium, and a 20 μ l aliquot diluted into 10 ml fresh medium and grown overnight. The phage was PEG precipitated as above, resuspended in 1 ml MPBS and loaded onto the column, washed and eluted as above.

Oligonucleotides synthesised:

CDR3PCR1 5'TGA GGA C(A or T) C(A or T)GC CGT CTA CTA CTG TGC
3'

Oligonucleotide probing

40 pmole oligonucleotide VH1FOR (Ward, E. S., et al (1989) Nature 341, 544-546), specific to pAb (D1.3) was phosphorylated with 100 μ Ci γ - 32 P ATP, hybridised (1pmole/ml) to nitrocellulose filters at 67°C in 6 x SSC buffer for 30 minutes and allowed to cool to room temperature for 30 mins, washed 3 x 1 min at 60°C in 0.1 x SSC.

Example 9

Construction of pAb Expressing Anti-hapten Activity

Oxazalone is a hapten that is a commonly used for studying

the detail of the immune response. The anti-oxazalone antibody, NQ11 has been described previously (E. Gherardi, R. Pannell, C. Milstein, J. Immunol. Method 126 61-68). A plasmid containing the VH and VL genes of NQ11 was converted to a ScFv form by inserting the BstEII/SacI fragment of SCFVD1.3 myc (nucleotides 432-499 of Fig 5) between the VH and VL genes to generate pSCFVNQ11, the sequence of which is shown in fig 13. This ScFv was cloned into the PstI/XhoI site of FdTPs/Xh (as described earlier) to generate pAb NQ11. (NQ11 has an internal PstI site and so it was necessary to do a complete digest of pSCFVNQ11 with XhoI followed by a partial digest with PstI)

The specific binding of pAb NQ11 was confirmed using ELISA. ELISA plates were coated at 37°C in 50mM NaHCO₃ at a protein concentration of 200 µg/ml. Plates were coated with either hen egg lysozyme (HEL), bovine serum albumin (BSA), or BSA conjugated to oxazalone (OX-BSA) (method of conjugation in Makela O., Kaartinen M., Pelkonen J.L.T., Karjalainen K. (1978) J.Exp.Med.148 1644). Preparation of phage, binding to ELISA plates, washing and detection was as described in example 6. Samples were assayed in duplicate and the average absorbance after 10 minutes presented in figure 14.

This result demonstrates that the pAb NQ11 binds the correct antigen. Figure 14 also shows that pAb D1.3 and pAb NQ11 bind only to the antigen against which the original antibodies were raised.

Example 10

Enrichment of pAb D1.3 from Mixtures of Other pAb by Affinity Purification

3×10^{10} phage in 10mls of PBSM at the ratios of pAb D1.3 to pAb NQ11 shown in table 2 were passed over a 1ml lysozyme Sepharose column. Washing, elution and other methods were as described in example 8 unless otherwise stated. Elutes from the columns were used to infect TG1 cells which were

then plated out. Colonies were probed with a probe which distinguishes pAb D1.3 from pAb NQ11. The sequence of this oligonucleotide (D1.3CDR3A) is:-

5'GTA GTC AAG CCT ATA ATC TCT CTC 3'

Table 2 presents the data from this experiment. An enrichment of almost 1000 fold was achieved in one round and an enrichment of over a million fold in two rounds of purification. This parallels the result described in example 8.

Example 11

Insertion of the Extracellular Domain of the Human Receptor for Platelet Derived Growth Factor Isoform BB into fdCAT-2

A gene fragment encoding the extracellular domain of the human receptor for platelet derived growth factor isoform BB (h-PDGFB-R) was isolated by amplification using the polymerase chain reaction, of plasmid RP41 (from the American Type Culture collection, Cat. No. 50735), a cDNA clone encoding amino acids 43 to 925 of the PDGF-B receptor (Gronwald, R.G.K. et al., PNAS 85, p3435-3439 (1988), amino acids 1 to 32 constitute the signal peptide). The oligonucleotide primers were designed to amplify the region of the h-PDGFB-R gene corresponding to amino acids 43 to 531 of the encoded protein. The primers also incorporate a unique ApaI site at the 5' end of the fragment and a unique XhoI site at the 3' end, to facilitate cloning into the vector fdCAT-2. The sequence of the primers is:

RPDGF1 5'C ACA GTG CAC GTC CTC AAT GTC TCC AGC ACC TTC 3'

RPDGF2 5'GAT CTC GAG CTT AAA GGG CAA GGA GTG TGG CAC 3'

PCR amplification was performed using high fidelity conditions (Eckert, K.A. and Kunkel, T.A., 1990 Nucl. Acids. Research 18 p3739-3744). The PCR mixture contained; 20mM Tris HCl (pH7.3 at 70°C), 50mM KCl, 4mM magnesium chloride, 0.01% gelatin, 1mM each of dATP, dGTP, dCTP, and dTTP, 500ng/ml RP41 DNA, 1µM each primer and 50 units/ml Taq polymerase (Cetus/Perkin-Elmer, Beaconsfield, Bucks, UK)

Thirty cycles of PCR were performed with denaturation at 92°C for 1 minute, annealing at 50°C for 2 min and extension at 72°C for 3 min. This reaction resulted in amplification of a fragment of ca 1500bp as expected.

fdCAT-2 vector DNA was digested with ApaI and XhoI (New England Biolabs) according to manufacturers recommendations, extracted with phenol/chloroform and ethanol precipitated (Sambrook et al., supra). Cloning of amplified RP41 DNA into this vector and identification of the desired clones was performed essentially as in example 12 except that probing was with 32-P labelled RPDGF1 and analytical PCR was performed using RPDGF1 and RPDGF2 as primers.

Example 12

Insertion of the Extracellular Domain of the Human Epidermal Growth Factor Receptor into fd-CAT2

A gene fragment encoding the extracellular domain of the human epidermal growth factor receptor (hEGF-R) was isolated by polymerase chain amplification of plasmid pJ3EGF-R (Clark et al. (1988) J. Cell Physiol. 134, p421-428) which contains the gene for hEGF-R (Ullrich, A. et al., Nature 309, p418-425, (1984)). The oligonucleotide primers used were designated to amplify the region of the hEGF-R gene corresponding to amino acid 1 of the mature protein through to amino acid 621. The primers also incorporate an unique ApaI site at the 5' end of the fragment and an unique Xho I site at the 3' end, to facilitate cloning into the vector fd-CAT2. The sequence of the primers is:

Oligo KSJ4: 5' GAT CTC GAG GGA CGG GAT CTT AAG CCC ATT CGT TGG 3'

Oligo KSJ5: 5' CAG AGT GCA CTG GAG GAA AAG AAA GTT TGC CA 3'

PCR amplification was carried out using high-fidelity conditions (Eckert, K.A. and Kunkel, T.A. 1990 Nucl. Acids Res. 18, 3739-3744). The PCR mixture contained: 20mM TrisHCl (pH 7.3 at 70°C), 50mM KCl, 4mM MgCl₂, 0.01%

gelatin, 1mM each of dATP, dGTP, dCTP and dTTP, 500ng/ml pJ3 plasmid, 0.5 μ M each primer and 50U/ml Taq polymerase (Cetus/Perkin-Elmer). Thirty cycles of PCR amplification were performed with denaturation at 92°C for 1 minute, annealing at 50°C for 2 minutes and extension at 72°C for 3 minutes. This regime resulted in amplification of a fragment of the expected size (approximately 1800bp).

The PCR mixture was extracted with phenol/chloroform and ethanol precipitated (Sambrook et al. supra.) before digestion with ApaI 1 and Xho 1 (New England BioLabs) according to manufacturers recommendations. The fragment was resolved on a 1% Tris-Acetate-EDTA agarose gel (Sambrook et al. supra.) and purified using Geneclean (BIO 101) (Geneclean, La Jolla, San Diego, California, USA) according to manufacturers recommendations.

fd-CAT2 vector DNA was digested with ApaI 1 and Xho 1 (New England BioLabs) according to manufacturers recommendations, extracted with phenol/chloroform and ethanol precipitated (Sambrook et al. supra.).

75ng of ApaI 1/Xho 1-digested vector DNA was ligated to 40ng of PCR-amplified ApaI 1/Xho I-digested hEGF-R fragment in 12 μ l of ligation buffer (66mM TrisHCl (pH7.6), 5mM MgCl₂, 5mM dithiothreitol, 100mg/ml bovine serum albumin, 0.5mM ATP, 0.5mM Spermidine) and 400 units T4 DNA ligase (New England BioLabs) for 16 hours at 16°C.

Two μ l of the ligation mixture was transformed into 200 μ l of competent E.coli MC1061 cells, plated on 2YT agar containing 15mg/ml tetracycline and incubated at 30°C for 20 hours.

Colonies containing hEGF-R were identified by probing with 32p-labelled KSJ 4 oligonucleotide (Sambrook et al. supra.) and the presence of an insert in hybridising colonies confirmed by PCR using the conditions described above. In this case the template DNA was prepared from the colonies by picking some colony material into 100 μ l of

distilled water and boiling for 10 minutes. 1µl of this mixture was used in a 20µl PCR.

Example 13.

Insertion of a Gene Encoding an Enzyme (Alkaline phosphatase) into fd-CAT2

As an example of the expression of a functional enzyme on the bacteriophage surface, the applicants have chosen bacterial alkaline phosphatase an enzyme that normally functions as a dimer (McCracken, S. and Meighen, E., J. Biol. Chem. 255, p2396-2404, (1980)). The oligonucleotides were designed to generate a PCR product with an Apa L1 site at the 5' end of phoA gene and a Not 1 site at its 3' end, thus facilitating cloning into fd-CAT 2 to create a gene III fusion protein. The oligonucleotides synthesised were:

phoA1:5' TAT TCT CAC AGT GCA CAA ACT GTT GAA CGG ACA CCA GAA ATG CCT GTT CTG 3' and,

phoA2:5' ACA TGT ACA TGC GGC CGC TTT CAG CCC CAG AGC GGC TTT C3'

The sequence of the phoA gene is presented in Chang C. N. et al., Gene 44, p121-125 (1986).

The PCR reaction was carried out in 100µl containing 50mM KCl, 2.5mM MgCl₂, 0.01% gelatin, 10mM Tris/HCl pH 8.3, 0.25 units/µl of Taq polymerase (Cetus/Perkin Elmer) and 0.5µg/ml template. The template was the pEK plasmid (described by Chaidaroglou et al., Biochemistry 27 p8338-8343, 1988). The PCR was carried out in a Techne (Techne, Duxford, Cambridge, UK) PHC-2 dri-block using thirty cycles of 1 min at 92°C, 2 min at 50°C, 3 min at 72°C.

The resultant product was extracted with phenol:chloroform, precipitated with ethanol, and the pellet dissolved in 35µl water. Digestion with 0.3 units/µl of Apl L1 was carried out in 150µl volume according to manufacturers instructions for two hours at 37°C. After heat inactivation of the enzyme at 65°C, NaCl was added to a final concentration of 150mM and 0.4 units/µl Not1 enzyme

added. After incubation for 2 hours at 37°C, the digest was extracted with phenol:chloroform and precipitated as above, before being dissolved in 30µl of water. The vector fd-CAT2 was sequentially digested with Apa L1 and NotI according to the manufacturers instructions and treated with calf intestinal alkaline phosphatase as described in example 2. The sample was extracted three times with phenol:chloroform, precipitated with ethanol and dissolved in water. The ligations were performed with a final DNA concentration of 1-2ng/µl of both the cut fd-CAT2 and then digested PCR product. The ligations were transformed into competent TG1 cells and plated on 2xTY tet plates. Identification of clones containing the desired insert was by analytical PCR performed using the conditions and primers above on boiled samples of the resulting colonies. The correct clone containing the phoA gene fused in frame to gene III was called fd-phoA1. The sequence at the junction of the cloning region is given in figure 15.

Example 14

Measuring Enzyme Activity of Phage-enzyme

Overnight cultures of TG1 or KS272 (E.coli cells lacking phoA. Strauch K. L., and Beckwith J. PNAS 85 1576-1580, 1988) cells containing either fd-phoA1 or fd-CAT2 were grown at 37°C in 2xTY with 15µg/ml tetracycline. Concentrated, PEG precipitated phage were prepared as described earlier. Enzyme assays (Malamy, M.H. and Horecker B.L., Biochemistry 3, p1893-1897, (1964)) were carried out at 24°C in a final concentration of 1M Tris/HCl pH 8.0, 1mM nitrophenyl phosphate (Sigma), 1mM MgCl₂. 100µl of a two times concentrate of this reaction mixture was mixed with 100µl of the test sample in a 96 well plate. Absorbance readings were taken every minute for 30 minutes at a wavelength of 405nm in a Titretek Mk 2 plate reader. Initial reaction rates were calculated from the rate of change of absorbance using a molar absorbance of 17000.

Standard curves (amount of enzyme vs. rate of change of absorbance) were prepared using dilutions of purified bacterial alkaline phosphatase (Sigma type III) in 10mM Tris/HCl pH 8.0, 1mM EDTA. The number of enzyme molecules in the phage samples were estimated from the actual rates of change of absorbance of the phage samples and comparison to this standard curve.

The results in Table 3 show that alkaline phosphatase activity was detected in PEG precipitated material in the sample containing fd-phoA1 but not fd-CAT2. Furthermore, the level of activity was consistent with the expected number of 1-2 dimer molecules of enzyme per phage. The level of enzyme activity detected was not dependant on the host used for growth. In particular, fd-phoA1 grown on phoA minus hosts showed alkaline phosphatase activity.

Therefore, the phage express active alkaline phosphatase enzyme from the phoA-gene III fusion on the phage surface.

Example 15

Insertion of Binding Molecules into Alternative Sites in the Phage

The availability of an alternative site in the phage for the insertion of binding molecules would open up the possibility of expressing more than one antibody fragment in a single pAb. This may be used to generate single or multiple binding specificities. The presence of two distinct binding activities on a single molecule will greatly increase the utility and specificity of this molecule. It may be useful in the binding of viruses with a high mutational rate such as human immunodeficiency virus. In addition, it may be used to bring antigens into close proximity (eg. drug targetting or cell fusion) or it may act as a "molecular clamp" in chemical, immunological or enzymatic processes.

The vector fd-tet and the derivatives described here, have a single BamH1 site in gene 3. This has previously

been used for the expression of peptide fragments on the surface of filamentous bacteriophage (Smith GP. (1985) *Science* 228 p1315-1317 and de la Cruz *et al.* (1988) *J Biol. Chem.* 263 p4318-4322). This provides a potential alternative site for the insertion of antibody fragments.

DNA fragments encoding SCFv's from D1.3 or NQ11 were generated by PCR using the primers shown below. These primers were designed to generate a fragment with BamH1 sites near both the termini, to enable cloning into the BamH1 site of gene3 (see figure 16(1)). The oligonucleotides used, also ensure that the resulting PCR product lacks Pst1 and Xho1 restriction sites normally used for manipulating the SCFv's (see figure 16(1)). This will facilitate subsequent manipulation of a second antibody fragment in the usual way at the N terminus of gene 3. The oligonucleotides used were:-

G3Bam1 5'TTT AAT GAG GAT CCA CAG GTG CAG CTG CAA GAG 3'

G3Bam2 5' AAC GAA TGG ATC CCG TTT GAT CTC AAG CTT 3'.

Preparation of vector and PCR insert

The PCR reaction was carried out in an 80 µl reaction as described in example 13 using 1ng/µl of template and 0.25U/µl of Taq polymerase and a cycle regime of 94°C for 1 minute, 60°C for 1 minute and 70°C for 2 minutes over 30 cycles. The template was either pSCFvNQ11 (example 9) or SCFvD1.3 myc (example 2). Reaction products were extracted with phenol:chloroform, precipitated, dissolved in water and digested with BamH1 according to manufacturers instructions. The digest was re-extracted with phenol:chloroform, precipitated and dissolved in water.

The vector FDTPs/Xh was cleaved with BamH1 and treated with calf intestinal phosphatase and purified as described in example 2. Ligations were set up at a vector concentration of approximately 6ng/µl and a PCR insert concentration of approximately 3ng/µl. These were ligated for 2.5 hours at room temperature before transforming into

competent TG1 cells and plating on TYE tet plates. The resultant colonies were probed as described in example 8. DNA was prepared from a number of colonies and the correct orientation and insert size confirmed by restriction digestion with Hind III in isolation or in combination with BamH1. (One Hind III site is contributed by one of the primers and the other by the vector).

Two clones containing a D1.3 insert (FDTBam1 and FDTBam2) and one containing an NQ11 insert (NQ11Bam1) were grown up and phage prepared as described earlier. ELISAs were carried out as described in example 6. No specific signal was found for any of these clones suggesting that the natural BamH1 site is not a suitable site for insertion of a functional antibody (results not shown).

It may be possible to clone into alternative sites to retain binding activity. The peptide repeats present in gene3 may provide such a site (figure 16 blocks A and B). This can be done by inserting a BamH1 site and using the PCR product described above. To facilitate this, the natural BamH1 site was removed by mutagenesis with the oligonucleotide shown below (using an in vitro mutagenesis kit (Amersham International)):-

G3mutδBam 5' CA AAC GAA TGG GTC CTC CTC ATT A 3'

The underlined residue replaces an A residue thereby removing the BamH1 site. DNA was prepared from a number of clones and several mutants lacking BamH1 sites identified by restriction digestion.

The oligonucleotide G3 Bamlink was designed to introduce a BamH1 site at a number of possible sites within the peptide linker sites A and B, see figure 16(2). The sequence of the linker is:

5'CC (G or A) CC ACC CTC GGA TCC (G or A) CC ACC CTC 3'

Its relationship to the peptide repeats in gene III is shown in figure 16.

Example 16PCR Assembly of Mouse VH and VL kappa (VLK) Repertoires
for Phage Display

5

The principle is illustrated in figure 17. Details are provided in sections A to F below but the broad outline is first discussed.

1. cDNA is prepared from spleen RNA from an appropriate mouse and the VH and VLK repertoires individually amplified. Separately, primers reverse and complementary to VH1FOR-2 (domain 1) and VLK2BACK (domain 2) are used to amplify an existing scFv-containing DNA by PCR. (The term FOR refers to eg. a primer for amplification of sequences on the sense strand resulting in antisense coding sequences. The term BACK refers to eg. a primer for amplification of sequences on the antisense strand resulting in sense coding sequences). This generates a 'linker' molecule encoding the linker with the amino acid sequence (1 letter code) (GGGGS)₃ which overlaps the two primary (VH and VLK) PCR products.
2. The separate amplified VH, VLK and linker sequences now have to be assembled into a continuous DNA molecule by use of an 'assembly' PCR. In the secondary 'assembly' PCR, the VH, VLK and linker

bands are combined and assembled by virtue of the above referred to overlaps, to generate an assembled PCR band with VHs and VLKs randomly spliced in frame for expression as scFVs.

5 The assembly PCR is carried out in two stages. Firstly, 7 rounds of cycling with just the three bands present in the PCR, followed by a further 20 rounds in the presence of the flanking primers VH1BACK (referring to domain 1 of VH) and VLKFOR.

10 The nucleotide sequences for these oligonucleotide primers are provided under the section entitled 'Primer Sequences' below. This two stage process, avoids the potential problem of preferential amplification of the first combinations to be assembled.

15 3. For cloning into the phage system, the assembled repertoires must be 'tagged' with the appropriate restriction sites. In the example provided below this is illustrated by providing an ApaL1 restriction site at the VH end of the continuous DNA molecule and a Not I site at the VLK end of the molecule. This is carried out by a third stage PCR using tagged primers. The nucleotide sequences for these oligonucleotide primers are also provided

20 under the section entitled 'Primer Sequences' below. There are however, 4 possible kappa light chain

25

sequences (whereas there is only a single heavy chain sequence). Therefore 4 oligonucleotide primer sequences are provided for VLK.

5 For this third stage PCR, sets of primers which have 4 and 10 nucleotides after the restriction sites have been used. However, long tags may give better cutting, in which case 15-20 nucleotide overhangs could be used.

 Scrupulously clean procedures must be used at all
10 times to avoid contamination during PCR. Negative controls containing no DNA must always be included to monitor for contamination. Gel boxes must be depurinated. A dedicated Geneclean kit (B10 101, Geneclean, La Jolla, San Diego, California, USA) can be
15 used according to manufacturers instructions to extract DNA from an agarose gel. The beads, NaI and the NEW wash should be aliquoted.

 All enzymes were obtained from CP Laboratories, P.O. Box 22, Bishop's Stortford, Herts CM20 3DH and the
20 manufacturers recommended and supplied buffers were used unless otherwise stated.

A. RNA Preparation

 RNA can be prepared using many procedures well known
25 to those skilled in the art. As an example, the following protocol (Triton X-100 lysis, phenol/SDS RNase

inactivation) gives excellent results with spleen and hybridoma cells (the addition of VRC (veronal ribosyl complex) as an RNase inhibitor is necessary for spleen cells). Guanidinium isothiocyanate/CsCl procedures
5 (yielding total cellular RNA) also give good results but are more time-consuming.

1. Wash $1 - 5 \times 10^7$ freshly harvested cells in 50 ml PBS at 800g for 10 minutes.
2. On ice, add 1 ml ice-cold lysis buffer (see later
10 for details) to the pellet and resuspend it with a 1 ml Gilson pipette. Leave on ice for 5 minutes.
3. Spin for 5 minutes at 4°C in a microfuge at 13000 rpm, in precooled tubes.
4. Transfer 0.5 ml of the supernatant to each of two
15 eppendorf tubes containing 60µl 10% (w/v) SDS and 250µl phenol (equilibrated with 100MM Tris-HCl pH 8.0). Vortex hard for 2 minutes, then microfuge (13000rpm) for five minutes at room temperature.
5. Re-extract the aqueous upper phase five times with
20 0.5 ml of phenol.
6. Precipitate with 1/10 volume 3M sodium acetate and 2.5 volumes ethanol at 20°C overnight or dry ice-isopropanol for 30 minutes.
7. Wash the RNA pellet and resuspend in 50µl water.
- 25 Use 2.5 to check concentration by measuring the optical density at 260nm and check 2µg on a 1% agarose gel. 40µg

to 107µg of RNA was obtained from spleen cells derived from mice.

Lysis buffer is [10mM Tris-HCl pH 7.4, 1mM MgCl₂,
5 150mM NaCl, 10mM VRC (New England Biolabs), 0.5% (w/v) Triton X-100], prepared fresh.

B. cDNA Preparation

cDNA can be prepared using many procedures well know to
10 those skilled in the art. As an example, the following protocol can be used:

1. Set up the following reverse transcription mix:

	<u>µl</u>
H ₂ O (DEPC-treated)	20
15 5mM dNTP	10
10 x first strand buffer	10
0.1M DTT	10
FOR primer(s) (10 pmol/µl)	2 (each) (see below)
RNasin (Promega; 40 U/µl)	4

20

NB

- i) DEPC is diethylpyrocarbonate, the function of which is to inactivate any enzymes that could degrade DNA or RNA
- 25 ii) dNTP is deoxynucleotide triphosphate
- iii) DTT is dithiothreitol the function of which is as an

antioxidant to create the anaerobic environment necessary for enzyme function.

iv) RNasin is a ribonuclease inhibitor obtained from Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA.

2. Dilute 10 µg RNA to 40 µl final volume with DEPC-treated water. Heat at 65°C for 3 minutes and hold on ice for one minute (to remove secondary structure).

3. Add to the RNA the reverse transcription mix (58 µl) and 4 µl of the cloned reverse transcriptase 'Super RT' (Anglian Biotech Ltd., Whitehall House, Whitehall Road, Colchester, Essex) and incubate at 42°C for one hour.

4. Boil the reaction mix for three minutes, cool on ice for one minute and then spin in a microfuge to pellet debris. Transfer the supernatant to a new tube.

10 x first strand buffer is [1.4M KCl, 0.5M Tris-HCl pH 8.1 at 42°C 80mM MgCl₂].

The primers anneal to the 3' end. Examples of kappa light chain primers are MJK1FONX, MJK2FONX, MJK4FONX and MJK5FONX (provided under 'Primer Sequences' below) and examples of heavy chain primers are CTG GAC AGG GAT CCA

GAG TTC CA and CTG GAC AGG GCT CCA TAG TTC CA. The two heavy chain primers are provided as alternatives, the four light chain primers are provided for kappa light chains 1-4. Primers annealing to CH1, VLK and VL domains could also be used.

C. Primary PCRs

For each PCR and negative control, the following reactions are set up. In the following, the Vent DNA polymerase sold by C.P. Laboratories Ltd. (New England Biolabs) address given above. The buffers are as provided by C.P. Laboratories.

	<u>μl</u>
H ₂ O	32.5
10 x Vent buffer	5
20 x Vent BSA	2.5
5mM dNTPs	1.5
FOR primer 10 pMol/μl)	2.5
BACK primer 10pmol/μl)	2.5

20

The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. For VH, the FOR primer is VH1FOR-2 and the BACK primer is VH1BACK. For VLK the FOR primers are MJK1FONX, MJK2FONX, MJK4FONX and MJK5FONX (for the four respective kappa light chains) and the BACK primer is VK2BACK. Only one kappa light chain BACK

primer is necessary, because binding is to a nucleotide sequence common to the four kappa light chains.

UV this mix 5 minutes. Add 2.5 μ l cDNA preparation (from B above), 2 drops paraffin oil (Sigma Chemicals, Poole, Dorset, UK). Place on a cycling heating block, e.g. PHC-2 manufactured by Techne Ltd. Duxford UK, pre-set at 94°C. Add 1 μ l Vent DNA polymerase under the paraffin. Amplify using 25 cycles of 94°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

Purify on a 2% lmp (low melting point agarose/TAE (tri-acetate EDTA) gel and extract the DNA to 20 μ l H₂O per original PCR using a Geneclean kit (see earlier) in accordance with the manufacturers instructions.

15 D. Preparation of linker

Set up in bulk (eg. 10 times):

	<u>μl</u>
H ₂ O	34.3
20 10 x Vent Buffer	5
20 x Vent BSA	2.5
5mM dNTPs	2
LINKFOR primer (10 pmol/ μ l)	2.5
LINKBACK primer (10 pmol/ μ l)	2.5
25 DNA from fcFv D1.3 (example 2)	1
Vent enzyme	0.2

The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. The FOR primer is LINKFOR and the BACK primer is LINKBACK.

- 5 Cover with paraffin and place on the cycling heating block (see above) at 94°C. Amplify using 25 cycles of 94°C 1 min, 65°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

10 Purify on 2% 1mp/TAE gel (using a loading dye without bromophenol blue as a 93bp fragment is desired) and elute with SPIN-X column (Costar Limited, 205 Broadway, Cambridge, Ma. USA.,) and precipitation. Take up in 5µl H₂O per PCR reaction.

15 E. Assembly PCRs

A quarter of each PCR reaction product (5µl) is used for each assembly. The total volume is 25µl.

For each of the four VLK primers, the following are set up:

20

H ₂ O	4.95
10 x Vent buffer	2.5
20 X Vent BSA	1.25
5mM dNTPs	0.8

- 25 UV irradiate this mix for 5 min. Add 5µl each of Vh and Vk band and 1.5µl of linker as isolated from the

preparative gels and extracted using the GeneClean kit as described in C and D above. Cover with paraffin. Place on the cycling heating block preset at 94°C. Add 1µl Vent under the paraffin. Amplify using 7 cycles of 94°C 2 min, 72°C 4 min. Then return the temperature to 94°C.

Add 1.5µl each of VH1BACK and the appropriate VKFOR primers MJK1FONX, MJK2FONX, MJK4FONX or MJK5FONX (10 pmol/µl) at 94°C. The primers should have been UV-treated as above. Amplify using 20 cycles of 94°C 1.5 min, 72°C 2.5 min. Post-treat at 60°C for 5 min. Purify on 2% 1mp/TAE gel and extract the DNA to 20µl H₂O per assembly PCR using a GeneClean kit (see earlier) in accordance with the manufacturers instructions.

15

F. Adding Restriction Sites

For each assembly and control set up:

		<u>µl</u>
20	H ₂ O	36.5
	10 x Taq buffer	5
	5mM dNTPs	2
	FOR primer (10 pmol/µl)	2.5
	BACK primer (10 pmol/µl)	2.5
25	Assembly product	1

The FOR and BACK primers are given in the section below entitled 'Primer Sequences'. The FOR primer is any of JK1NOT10, JK2NOT10, JK4NOT10 or JK5NOT10 (for the four respective kappa light chains) for putting a NotI
 5 restriction site at the VLK end. The BACK primer is HBKAPA10 for putting an ApaI restriction site at the VH end.

Cover with paraffin and place on the cycling heating block preset at 94°C. Add 0.5 µl Cetus Taq DNA
 10 polymerase (Cetus/perkin-Elmer, Beaconsfield, Bucks, UK) under the paraffin. Amplification is carried out using 11 to 15 rounds of cycling (depends on efficiency) at 94°C 1 min, 55°C 1 min, 72°C 2 min. Post-treat at 60°C for 5 min.

15 10 x Taq buffer is [0.1M Tris-HCl pH 8.3 at 25°C, 0.5M KCl, 15mM MgCl₂, 1mg/ml gelatine].

G. Work-up

Purify once with CHCl₃/IAA (isoamylalcohol), once
 20 with phenol, once with CHCl₃/IAA and back-extract everything to ensure minimal losses. Precipitate and wash twice in 70% EtOH. Dissolve in 70µl H₂O.

Digest overnight at 37°C with NotI:	µl
25 DNA (joined seq)	70
NEB NotI buffer x 10	10

NEB BSA x 10 10

Not1 (10 U/ μ l) 10

The DNA (joined sequence) above refers to the assembled DNA sequence comprising in the 5' to 3' direction

5 ApaL1 restriction site

VH sequence

Linker sequence

VLK sequence

Not 1 restriction site.

10 The VLK sequence may be any one of four possible kappa chain sequences.

 The enzymes Not 1 above, ApaL1 below and the buffers NEB Not 1, NEB BSA above and the NEB buffer 4 (below) are obtainable from CP Laboratories, New England Biolabs
15 mentioned above.

 Re-precipitate, take up in 80 μ l H₂O. Add to this 10 μ l NEB buffer 4 and 10 μ l ApaL 1.

 Add the enzyme ApaL1 in aliquots throughout the day, as it has a short half-life at 37°C.

20 Purify on 2% lmp/TAE gel and extract the DNA using a GeneClean kit, in accordance with the manufacturers instructions. Redigest if desired.

F. Final DNA product

25 The final DNA product is an approximate 700 bp fragment with Apa L1 and Not1 compatible ends consisting

of randomly associated heavy and light chain sequences linked by a linker. A typical molecule of this type is the scFvD1.3 molecule incorporated into fdscFvD1.3 described in example 3. These molecules can then be
 5 ligated into suitable fd derived vectors, e.g. fdCAT2 or fd CAT3 (example 1), using standard techniques.

Primer sequences.

10 Primary PCR oligos (restriction sites underlined):

VH1FOR-2 TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC

VH1BACK AGG TSM ARC TGC AGS AGT CWG G

MJK1FONX CCG TTT GAT TTC CAG CTT GGT GCC

MJK2FONX CCG TTT TAT TTC CAG CTT GGT CCC

15 MJK4FONX CCG TTT TAT TTC CAA CTT TGT CCC

MJK5FONX CCG TTT CAG CTC CAG CTT GGT CCC

VK2BACK GAC ATT GAG CTC ACC CAG TCT CCA

PCR oligos to make linker:

20 LINKFOR TGG AGA CTC GGT GAG CTC AAT GTC

LINKBACK GGG ACC ACG GTC ACC GTC TCC TCA

For adding restriction sites:

HBKAPA10 CAT GAC CAC AGT GCA CAG GTS MAR CTG CAG SAG TCW

25 GG

JK1NOT10 GAG TCA TTC TGC GGC CGC CCG TTT GAT TTC CAG CTT

GGT GCC

JK2NOT10 GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAG CTT

GGT CCC

JK4NOT10 GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAA CTT

5 TGT CCC

JK5NOT10 GAG TCA TTC TGC GGC CGC CCG TTT CAG CTC CAG CTT

GGT CCC

Example 17

10 Insertion of the Extracellular Domain of the Human
Receptor for Platelet Derived Growth Factor PDGF isoform
BB into fdCAT2

This example is substantially equivalent to example 11. However, in example 11, amino acids 33-42 of the mature protein were not provided. In this example, the primer RPDGF3 also includes bases encoding these amino acids.

A gene fragment encoding the extracellular domain of the human receptor for platelet derived growth factor isoform BB (h-PDGFB-R) was isolated by amplification, using the polymerase chain reaction, of plasmid RP41 (from the American Type Culture collection, Cat. No. 50735), a cDNA clone encoding amino-acids 43 to 925 of the PDGF-B receptor (Gronwald, R.G.K. et al PNAS 85 p3435-3439 (1988), amino acids 1 to 32 constitute the signal peptide). The oligonucleotide primers were

designed to amplify the region of the h-PDGFB-R gene corresponding to amino acids 43 to 531 of the encoded protein. The primer RPDGF3 for the N-terminal region also included bases encoding amino acids 33 to 42 of the h-PDGFB-R protein (corresponding to the first ten amino acids from the N-terminus of the mature protein) to enable expression of the complete extracellular domain. The primers also incorporate a unique ApaI site at the N-terminal end of the fragment and a unique XhoI site at the C terminal end to facilitate cloning into the vector fdCAT2. The sequence of the primers is:

RPDGF3 5' CAC AGT GCA CTG GTC GTC ACA CCC CCG GGG CCA GAG
CTT GTC CTC AAT GTC TCC AGC ACC TTC GTT CTG 3'

15

RPDGF2 5' GAT CTC GAG CTT AAA GGG CAA GGA GTG TGG CAC 3'

PCR amplification was performed using high fidelity conditions (Eckert, K.A. and Kunkel, T.A. 1990 Nucl Acids Research 18 3739-3744). The PCR mixture contained: 20mM TrisHCl (pH7.3 at 70°C, 50mM KCl, 4mM magnesium chloride, 0.01% gelatin, 1mM each of dATP, dCTP, dGTP and dTTP, 500ng/ml RP41 DNA, 1µM each primer and 50units/ml Taq polymerase (Cetus/Perkin Elmer, Beaconsfield, Bucks, U.K.). Thirty cycles of PCR were performed with denaturation at 92°C for 1 min, annealing at 60°C for 1

25

min and extension at 72°C for 1.5 min. This reaction resulted in amplification of a fragment of ca. 1500bp as expected.

fdCAT2 vector DNA (see example 5) was digested with
 5 ApaI and XhoI (New England Biolabs) according to
 manufacturers recommendations, extracted with
 phenol/chloroform and ethanol precipitated (Sambrook et
 al, supra). Cloning of amplified RP41 DNA into this
 vector and identification of the desired clones was
 10 performed essentially as in example 12 except that
 probing was with 32-P labelled RPDGF2 and analytical PCR
 was performed using RPDGF3 and RPDGF2.

Example 18

Binding of 125 I-PDGF-BB to the Extracellular Domain of
 the Human Receptor for Platelet Derived Growth Factor
 Isoform BB Displayed on the Surface of fd Phage.
 5 Measured using an Immunoprecipitation Assay.

Phage particles, expressing the extracellular domain
 of the human platelet derived growth factor isoform BB
 receptor (fd h-PDGFB-R), were prepared by growing E.coli
 MC1061 cells transformed with fd h-PDGFB-R in 50ml of
 10 2xTY medium with 15ug/ml tetracyclin for 16 to 20 hours.
 Phage particles were concentrated using polyethylene
 glycol as described in example 6 and resuspended in PDGF
 binding buffer (25mM HEPES, pH7.4, 0.15mM NaCl, 1mM

magnesium chloride, 0.25% BSA) to 1/33rd of the original volume. Residual bacteria and undissolved material were removed by spinning for 2 min in a microcentrifuge. Immunoblots using an antiserum raised against gene III (Prof. I. Rashed, Konstanz., Germany) show the presence in such phage preparations of a geneIII-h-PDGFB-R protein of molecular mass 125000 corresponding to a fusion between h-PDGFB-R external domain (55000 daltons) and geneIII (apparent molecular mass 70000 on SDS-polyacrylamide gel).

Duplicate samples of 350µl concentrated phage were incubated with ^{125}I -PDGF-BB (78.7fmol, 70nCi, 882Ci/mmol; Amersham International plc, Amersham, Bucks) for 1 hour at 37°C. Controls were included in which fdTPs/Bs vector phage (figure 4(26)) or no phage replaced fd h-PDGFB-R phage. After this incubation, 10ul of sheep anti-M13 polyclonal antiscrum (a gift from M. Hobart) was added and incubation continued for 30 min at 20°C. To each sample, 40ul (20ul packed volume) of protein G Sepharose Fast Flow (Pharmacia, Milton Keynes) equilibrated in PDGF binding buffer was added. Incubation was continued for 30 min at 20°C with mixing by end over end inversion on a rotating mixer. The affinity matrix was spun down in a microcentrifuge for 2 min and the supernatant removed by aspiration. Non-specifically bound ^{125}I -PDGF-BB was removed by resuspension of the pellet in 0.5ml PDGF

binding buffer, mixing by rotation for 5 min, centrifugation and aspiration of the supernatant, followed by two further washes with 0.5ml 0.1% BSA, 0.2% Triton-X-100. The pellet finally obtained was
5 resuspended in 100ul PDGF binding buffer and counted in a Packard gamma counter. For displacement studies, unlabelled PDGF-BB (Amersham International) was added to the stated concentration for the incubation of ^{125}I -PDGF-BB with phage.

10 ^{125}I -PDGF-BB bound to the fd h-PDGFB-R phage and was immunoprecipitated in this assay. Specific binding to receptor phage was 3.5 to 4 times higher than the non-specific binding with vector phage fdTPs/Bs or no phage (fig 19). This binding of ^{125}I -PDGF-BB could be
15 displaced by the inclusion of unlabelled PDGF-BB in the incubation with phage at 37°C (fig 20). At 50nM, unlabelled PDGF-BB the binding of ^{125}I -PDGF-BB was reduced to the same level as the fdTPs/Bs and no phage control. Figure 21 shows the same data, but with the
20 non-specific binding to vector deducted.

These results indicate that a specific saturable site for ^{125}I -PDGF-BB is expressed on fd phage containing cloned h-PDGFB-R DNA. Thus, the phage can display the functional extracellular domain of a cell surface
25 receptor.

Example 19, Construction of Binding molecule-Gene III
Phagemid

It would be useful to improve the transfection efficiency of the phage-binding molecule system and also
5 to have the possibility of displaying different numbers and specificities of binding molecules on the surface of the same bacteriophage. The applicants have devised a method that achieves both aims.

The approach is derived from the phagemid system
10 based on pUC119 [Vieira, J and Messing, J. (1987) Methods Enzymol. 153:3]. In brief, gene III from fd-CAT2 (example 5) and fd-CAT2 ScFv D1.3 (example 2) was cloned downstream of the lac promoter in pUC119 in order that the mutated gene III could be 'rescued' by M13K07 helper
15 phage [Vieira, J and Messing, J. et supra.]. The majority of rescued phage would be expected to contain a genome derived from the pUC119 plasmid that contains the binding molecule-gene III fusion and should express varying numbers of the binding molecule on the surface up
20 to the normal maximum of 3-5 molecules of gene III on the surface of wild type phage. The system has been exemplified below using an antibody as the binding molecule.

An fdCAT2 containing the single chain Fv form of the
25 D1.3 antilysozyme antibody was formed by digesting FDTSCFVD1.3 (example 2) with PstI and XhoI, purifying the

fragment containing the scFv fragment and ligating this into PstI and XhoI digested fdCAT2. The appropriate clone, called fdCAT2 scFvD1.3 was selected after plating onto 2xTY tetracyclin (15µg/ml) and confirmed by restriction enzyme and sequence analysis.

Gene III from fd-CAT2 (example 5) and fd-CAT2 ScFv D1.3 was PCR-amplified using the primers shown below:

Primer A: TGC GAA GCT TTG GAG CCT TTT TTT TTG GAG ATT TTC AAC G

10 Primer B: CAG TGA ATT CTT ATT AAG ACT CCT TAT TAC GCA GTA TGT TAG C

Primer A anneals to the 5' end of gene III including the ribosome binding site and incorporates a Hind III site. Primer B anneals to the 3' end of gene III at the C-terminus and incorporates two UAA stop codons and an EcoRI site. One hundred ng of fd-CAT2 and fd-CAT2 ScFv D1.3 DNA was PCR-amplified in a total reaction volume of 50µl as described in example 11, except that 20 cycles of amplification were performed: 94°C 1 minute, 50°C 1 minute, 72°C 3 minutes. This resulted in amplification of the expected 1.2Kb fragment from fd-CAT2 and a 1.8Kb fragment from fd-CAT2 ScFv D1.3.

The PCR fragments were digested with EcoRI and Hind III, gel-purified and ligated into EcoRI- and Hind III-cut and dephosphorylated pUC119 DNA and transformed into E.coli TG1 using standard techniques (Sambrook et al., et

supra). Transformed cells were plated on SOB agar containing 100µg/ml ampicillin and 2% glucose. The resulting clones were called pCAT-3 (derived from fd-CAT2) and pCAT-3 ScFv D1.3 (derived from fd-CAT2 ScFv D1.3).

Example 20, Rescue of Anti-Lysozyme Antibody Specificity from pCAT-3 ScFv D1.3 by M13K07

Single CAT-3 and CAT-3 ScFv D1.3 colonies were picked into 1.5ml 2YT containing 100µg/ml ampicillin and 2% glucose, and grown 6hrs at 30°C. 30µl of these stationary cells were added to 6mls 2YT containing 100µg/ml ampicillin and 2% glucose in 50ml polypropylene tubes (Falcon, Becton Dickinson Labware, 1950 Williams Drive, Oxnard, CA. USA) and grown for 1.5hrs at 30°C at 380rpm in a New Brunswick Orbital Shaker (New Brunswick Scientific Ltd., Edison House 163 Dixons Hill Road, North Mimms. Hatfield, UK). Cells were pelleted by centrifugation at 5,000g for 25 minutes and the tubes drained on tissue paper. The cell pellets were then resuspended in 6mls 2YTamp containing 100µg/ml ampicillin (no glucose) and 4mls 2YT containing 1.25×10^9 p.f.u. ml⁻¹ M13K07 bacteriophage added. The mixture was left on ice for 5 minutes followed by growth at 35°C for 45 minutes at 450rpm. A cocktail was then added containing 4µl 100µg/ml ampicillin, 0.5µl 0.1M IPTG and 50µl 10mg/ml

Kanamycin, and the cultures grown overnight at 35°C, 450rpm.

The following day the cultures were centrifuged and phage particles PEG precipitated as described in example 6. Phage pellets were resuspended in 100µl TE (tris-EDTA see example 6) and phage titred on E.coli TG1. Aliquots of infected cells were plated on 2YT containing either 100µg/ml ampicillin to select for pUC119 phage particles, or 50µg/ml Kanamycin to select for the M13 K07 helper phage. Plates were incubated overnight at 37°C and antibiotic-resistant colonies counted:

DNA	amp ^R	Kan ^R
pCAT-3	1.8x10 ¹¹ colonies	1.2x10 ⁹ colonies
pCAT-3ScFv D1.3	2.4x10 ¹¹ colonies	2.0x10 ⁹ colonies

This shows that the amp^R phagemid particles are infective and present in the rescued phage population at a 100-fold excess over Kan^R M13K07 helper phage.

Phage were assayed for anti-lysozyme activity by ELISA as described in example 6, with the following modifications:

- 1) ELISA plates were blocked for 3 hrs with 2% Marvel/PBS
- 2) 50µl phage, 400µl 1xPBS and 50µl 20% marvel were mixed end over end for 20 minutes at room temperature before adding 150µl per well.
- 3) Phage were left to bind for 2 hours at room

temperature.

4) All washes post phage binding were:

2 quick rinses PBS/0.5% TWEEN 20

3x2 minute washes -----"

5 2 quick rinses PBS no detergent

3x2 minute washes -----"

The result of this ELISA is shown in figure 22, which shows that the antibody specificity can indeed be rescued efficiently.

10 It is considered a truism of bacterial genetics that when mutant and wild-type proteins are co-expressed in the same cell, the wild-type protein is used preferentially. This is analagous to the above situation wherein mutant (i.e. antibody fusion) and wild-type gene
15 III proteins (from M13K07) are competing for assembly as part of the pUC119 phagemid particle. It is therefore envisaged that the majority of the resulting pUC 119 phage particles will have fewer gene III-antibody fusion molecules on their surface than is the case for the
20 purely phage system described for instance in example 2. Such phagemid antibodies are therefore likely to bind antigen with a lower avidity than fd phage antibodies with three or more copies of the antibody fusion on their surfaces (there is no wild-type gene III protein in the
25 system described, for instance, in example 2), and provide a route to production of phage particles with

different numbers of the same binding molecule (and hence different avidities for the ligand/antigen) or multiple different binding specificities on their surface, by using helper phage such as M13K07 to rescue cells
5 expressing two or more gene III-antibody fusions.

It is also possible to derive helper phage that do not encode a functional gene III in their genomes (by for example deleting the gene III sequence or a portion of it or by incorporating an amber mutation within the gene).
10 These defective phages will only grow on appropriate cells (for example that provide functional gene III in trans, or contain an amber suppressor gene), but when used to rescue phage antibodies, will only incorporate the gene III antibody fusion encoded by the phagemid into
15 the released phage particle.

Example 21. Transformation Efficiency of pCAT-3 and pCAT-3 ScFv D1.3 phagemids

PUC 19, pCAT-3 and pCAT-3 ScFv D1.3 plasmid DNAs,
20 and fdCAT-2 phage DNA was prepared, and used to transform E.coli TG1, pCAT-3 and pCAT-3 ScFv D1.3 transformations were plated on SOB agar containing 100µg/ml ampicillin and 2% glucose, and incubated overnight at 30°C. fdCAT-2 transformations were plated on 2YT agar containing
25 15µg/ml tetracycline and incubated overnight at 37°C. Transformation efficiencies are expressed as colonies per

µg of input DNA.

	DNA	Transformation efficiency
	pUC 19	1.10^9
5	pCAT-3	1.10^8
	pCAT-3ScFv D1.3	1.10^8
	fd CAT-2	8.10^5

As expected, transformation of the phagemid vector is approximately 100-fold more efficient than the parental fdCAT-2 vector. Furthermore, the presence of a ScFv antibody fragment does not compromise efficiency. This improvement in transformation efficiency is practically useful in the generation of phage antibody libraries that have large repertoires of different binding specificities.

Table 1. Enrichment of pAb (D1.3) from vector population

INPUT RATIO ^a	OUTPUT RATIO		ENRICHMENT ^d
	oligo ^b	ELISA ^c	
pAb : fd-CAT1	pAb : total phage	pAb : total phage	
Single Round			
1 : 4x10 ³	43/124		1.3 x 10 ³
1 : 4x10 ⁴	2/82		1.0 x 10 ³
Two Rounds			
1 : 4x10 ⁴	197/372		2.1 x 10 ⁴
1 : 4x10 ⁵	90/356	3/24	1.0 x 10 ⁵
1 : 4x10 ⁶	27/183	5/26	5.9 x 10 ⁵
1 : 4x10 ⁷	13/278		1.8 x 10 ⁶

Footnotes: ^aApproximately 10¹² phage with the stated ratio of pAb (D1.3) : FDTPs/Bs were applied to 1 ml lysozyme-sepharose columns, washed and eluted. ^bTG1 cells were infected with the eluted specific binding phage and plated onto TY-tet plates. After incubation overnight at 30-37°C the plates were analysed by hybridisation to the ³²P-labelled oligonucleotide VH1FOR (Ward et al op cit) which is specific to pAb D1.3. ^cSingle colonies from overnight plates were grown, phage purified, and tested for lysozyme binding. ^dEnrichment was calculated from the oligonucleotide probing data.

Table 2 Enrichment of pAb(D1.3) from mixed pAb population

Input Ratio ¹ (pAbD1.3:pAbNQ11)	Output Ratio ² (pAb D1.3: Total phage)	Enrichment
Single Round		
1 : 2.5 x 10 ⁴	18/460	0.98 x 10 ³
1 : 2.5 x 10 ⁵	3/770	0.97 x 10 ³
1 : 2.5 x 10 ⁶	0/112	-
pAb NQ11 only	0/460	-
Second Round		
1 : 2.5 x 10 ⁴	119/170	1.75 x 10 ⁴
1 : 2.5 x 10 ⁵	101/130	1.95 x 10 ⁵
1 : 2.5 x 10 ⁶	102/204	1.26 x 10 ⁶
1 : 2.5 x 10 ⁷	0/274	-
1 : 2.5 x 10 ⁸	0/209	-
pAb NQ11 only	0/170	-

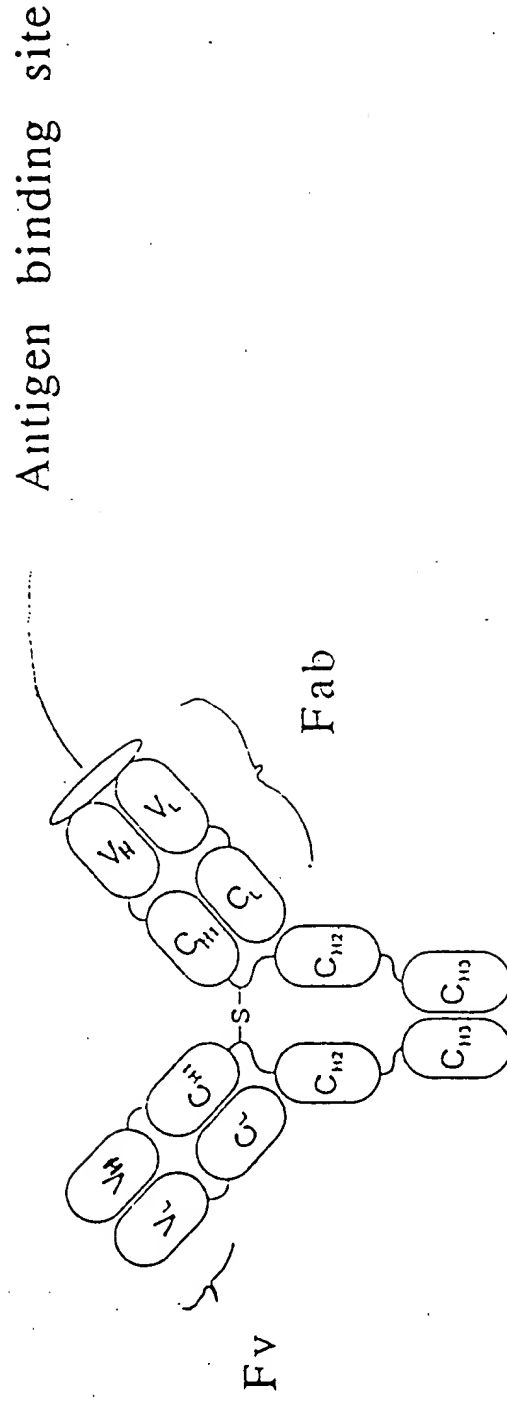
Notes

1. 10¹⁰ phage applied to a lysozyme column as in table 1.
2. Plating of cells and probing with oligonucleotide as in table 1, except the oligonucleotide was D1.3CDR3A

Table 3: Enzymic activity of phage-enzyme

Input	ng of enzyme or No. of phage	Rate (OD/hr)	No. of molecules of Enzyme equivalent ($\times 10^{-11}$)
Pure Enzyme	335	34	24.5
Pure Enzyme	177.5	17.4	12.25
Pure Enzyme	88.7	8.7	6.125
Pure Enzyme	44.4	4.12	3.06
Pure Enzyme	22.2	1.8	1.5
Pure Enzyme	11.1	0.86	0.76
No Enzyme	0	0.005	0
fd-phoA1/TG1	1.83×10^{11}	5.82	4.2
fd-CAT2/TG1	1.0×10^{12}	0.155	0.112
fd-phoA1/KS272	7.1×10^{10}	10.32	7.35
fd-CAT2/KS272	8.2×10^{12}	0.038	0.027

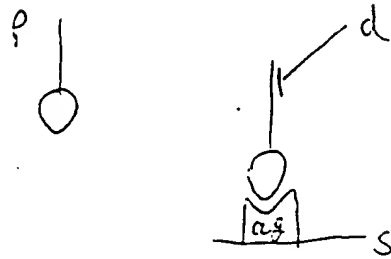
FIGURE 1: Antibody structure



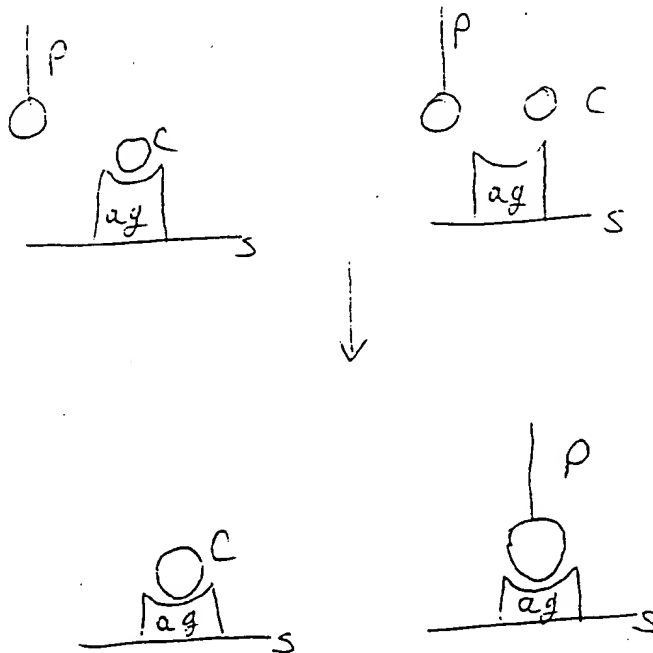
The single domain antibody (dAb, Ward et al. 1989) consists of a single V_H domain.

FIGURE 2: ASSAY FORMATS

2 i) Binding/elution



2 ii) Competition



- P - Phage antibody population to be sampled.
- ag - Antigen to which binding required.
- c - Competitor antibody/ phAb/ligand etc population.
- s - Surface (eg plastic, beads etc).
- d - Detection system

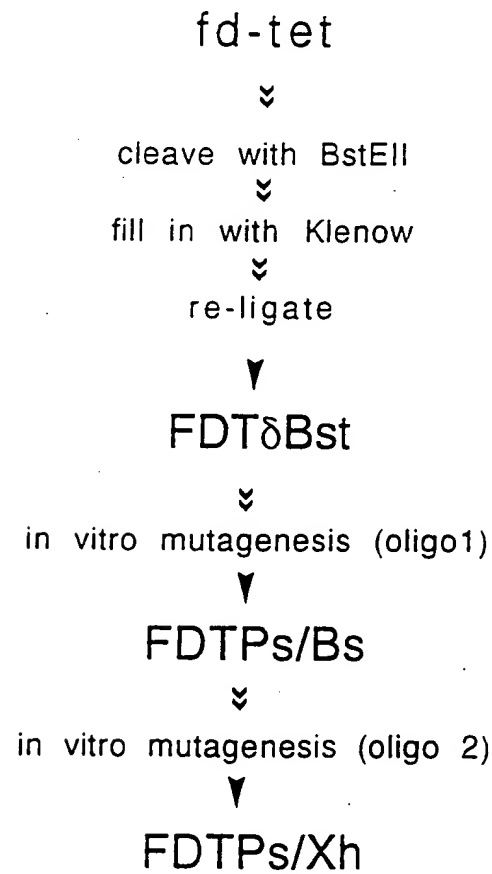
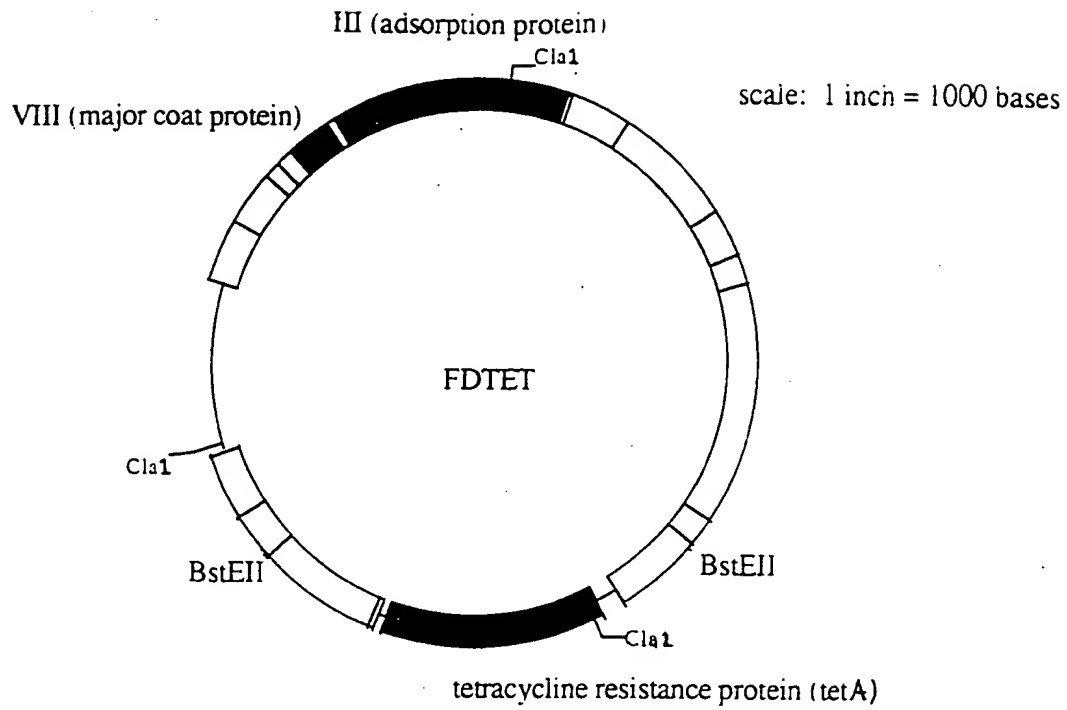


Figure 3 Scheme for construction of vectors

1

(1653)
Oligo 1 ACA ACT TTC AAC AGT TGA GGA GAC GGT GAC CGT AAG CTT CTG CAG TTG GAC CTG AGC
GGA GTG AGA ATA (1620)

(1653)
Oligo 2 ACA ACT TTC AAC AGT TTC CCG TTT GAT CTC GAG CTC CTG CAG TTG GAC CTG

(1704)
Oligo 3 GTC GTC TTT CCA GAC GTT AGT

2

GENE III

GENE III

SIGNAL
CLEAVAGE SITE

(1624)
A TCT CAC TCC GCT _____

(1650)
GAA ACT GTT GAA AGT

Q V Q L Q V T V S S
B TCT CAC TCC GCT CAG GTC CAA CTG CAG AAG CTT ACG GTC ACC GTC TCC TCA ACT GTT GAA AGT
PstI BstEII

Q V Q L Q L E I K R
C TCT CAC TCC GCT CAG GTC CAA CTG CAG GAG CTC GAG ATC AAA CGG GAA ACT GTT GAA AGT
PstI XhoI

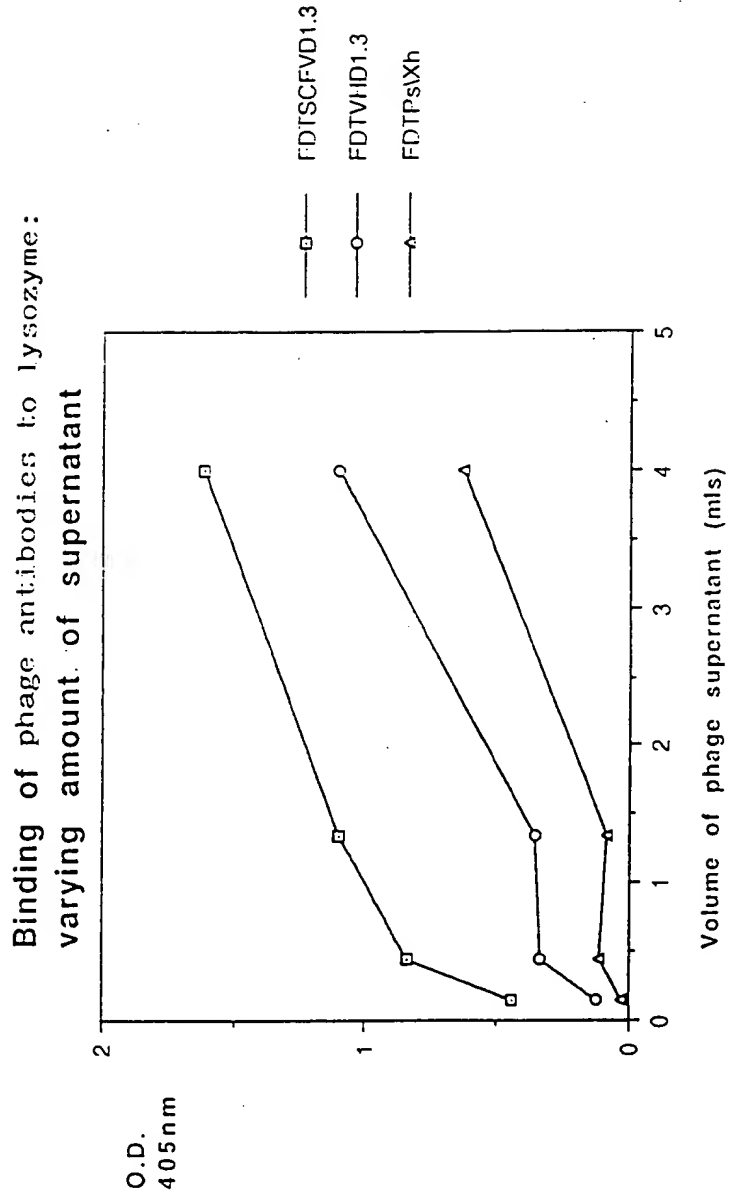
B = FDTPs/Bs
C = FDTPs/Xh

Figur 4. Sequence of oligos and vectors

rbs M K V L I E T A A
 GCATGCAAATTCATTTCAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCC
 10 20 30 40 50 60
 SphI
 PelB leader
 A G L L L L A A O P A M E Q V Q L Q E S
 GCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCCGAGGTGCAGCTGCAGGAGTCA
 70 80 90 100 110 120
 PstI
 G P G L V A P S Q S L S I T C T V S G F
 GGACCTGGCCCTGGTGGCGCCCTCACAGAGCCTGTCCATCAGATGCACCGTCTCAGGGTTC
 130 140 150 160 170 180
 S L T G Y G V N W V R Q P P G K G L E W
 TCATTAAACCGGTATGGTGTAAACTGGGTTGCCAGCCTCCAGGAAAGGGTCTGGAGTGG
 190 200 210 220 230 240
 VED1.3
 L G M I W S D G H T D Y H S A L K S R L
 CTGGGAATGATTTCGGGTGATGGAAACACAGACTATAATTCAGCTCTCAAATCCAGACTG
 250 260 270 280 290 300
 S I S K D H S K S Q V F L H M H S L H T
 AGCATCAGCAAGGACAACTCCAGAGCCCAAGTTTCTTAAAAATGAACAGTCTGCACACT
 310 320 330 340 350 360
 D D T A R Y Y C A E E R D Y R L D Y W G
 GATGACACAGCCAGGTACTACTGTGCCAGAGAGAGAGATTATAGGCTTGACTACTGGGGC
 370 380 390 400 410 420
 Linker Peptide
 Q Q T T V T V S S R R R R S S G G R R S S
 CAAGGCCACCACGGTCACTCTCTCAGgtggaggcgggttcaggcggagggtgggtctggc
 430 440 450 460 470 480
 BstEII
 R R G S D I E L T Q S P A S L S A S V G
 ggtggcggatcgGACATCGAGCTCACTCACTCTCCAGCCTCCCTTTCTGCGTCTGTGGGA
 490 500 510 520 530 540
 SacI
 E T V T I T C R A S G H I H N Y L A W Y
 GAAACTGTCAACATCACATGTTCGAGCAAGTGGGAATATTCACAATTATTTAGCATGGTAT
 550 560 570 580 590 600
 Q Q K Q G K S P Q L L V Y Y T T T L A D
 CAGCAGAAACAGGGAATCTCTCTCAGCTCCTGGTCTATTATACAACAACCTTAGCAGAT
 610 620 630 640 650 660
 VKD1.3
 S V P S R F S G S G S G T Q Y S L K I H
 GGTGTGCCATCAAGGTTTCAGTGGCAGTGGATCAGGAACACAATATTCTCTCAAGATCAAC
 670 680 690 700 710 720
 S L Q P E D F G S Y Y C Q H F W S T P R
 AGCCTGCAACCTGAAGATTTTGGGAGTTTATTACTGTCAACATTTTGGAGTACTCCTCGG
 730 740 750 760 770 780
 Myc Tag (TAG1)
 T F G G G T K L E I K R E Q K L I S E E
 ACGTTCGGTGGAGGCCACCAAGCTCGAGATCAAAACGGGAACAAAACTCATCTCAGAAGAG
 790 800 810 820 830 840
 XhoI
 D L H * *
 GATCTGAATTAAATGATCAAAACGGTAATAAGGATCCAGCTCGAATTC
 850 860 870 880
 EcoRI

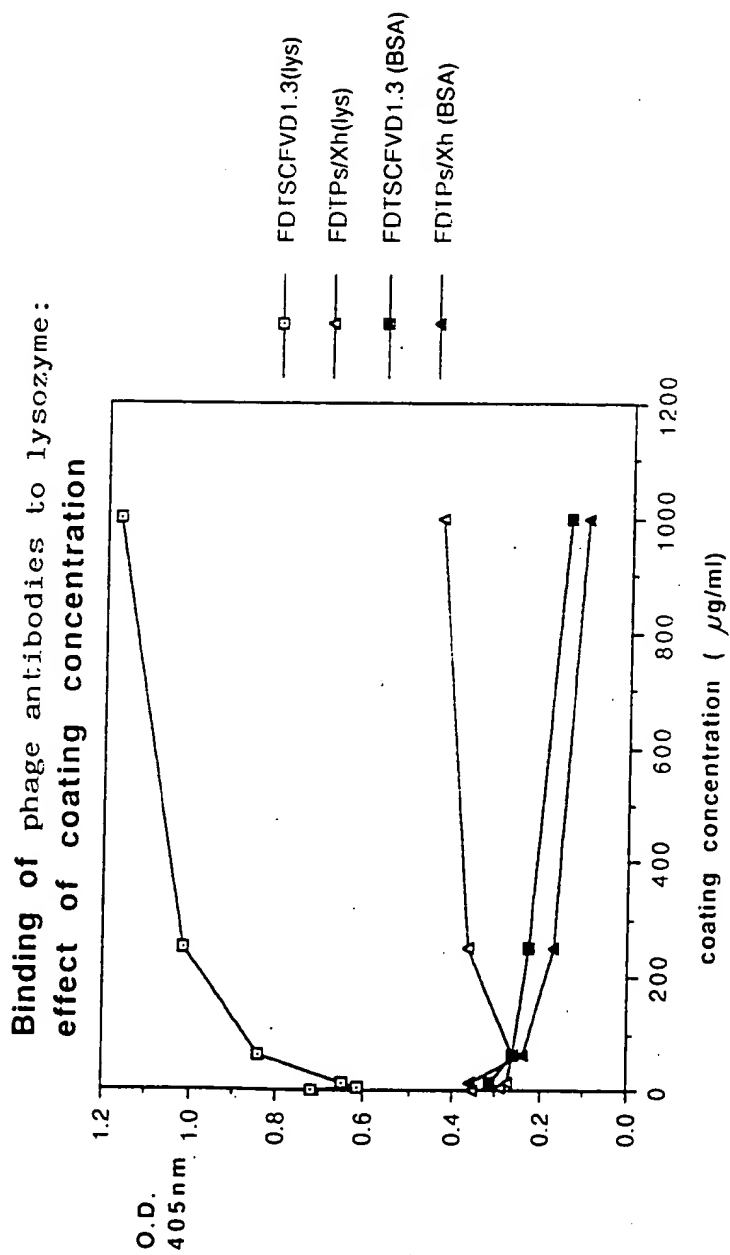
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Fig. 6.



Methods as described in example 4

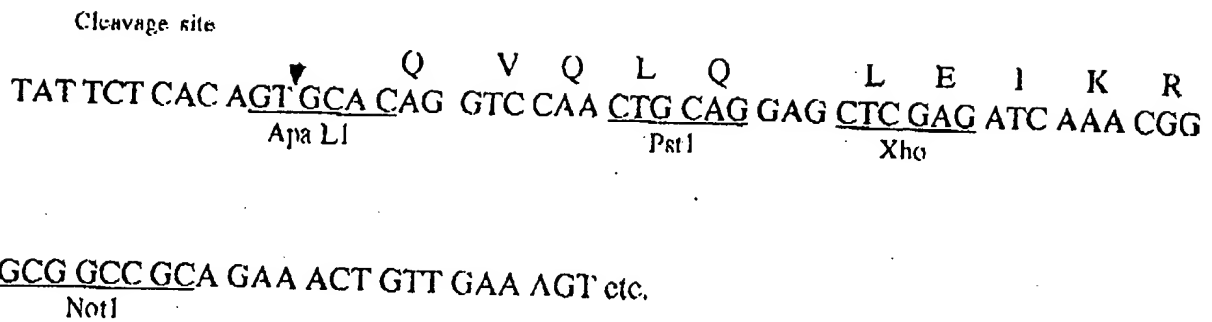
Fig. 7



Methods as described in example 4

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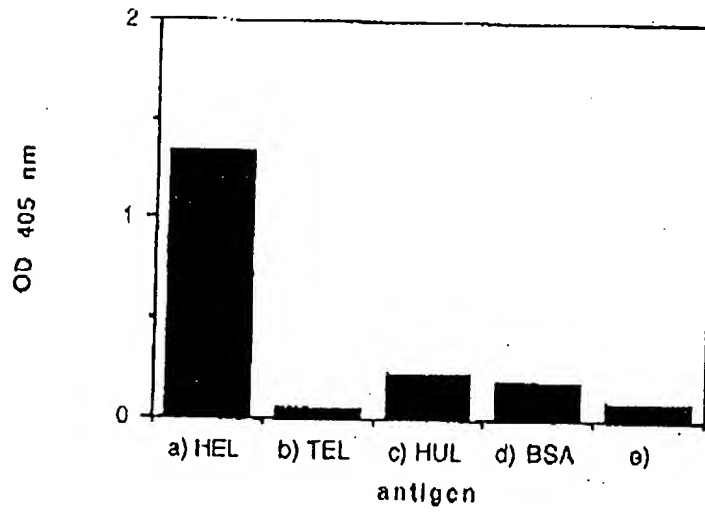
Fig. 8. Sequence around the cloning site of fd-CAT2.



Restriction enzyme sites are shown as well as the amino acids encoded by antibody derived sequences. These are flanked at the 5' end by the gene 3 signal peptide and at the 3' end by 3 alanine residues (encoded by the Not I restriction site) and the remainder of the mature gene 3 protein.

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Figure 9 Binding of pAb(D1.3) to lysozymes



Figur 10: S qu nc of Fab D1.3

M K Y L L P T A A A G L L L P A A Q P A
 CATGCAAATTCATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACCTGCTGCCCCAACCAG
 11 21 31 41 51 61 71 81 91

M A Q V Q L Q E S G P G L V A P S Q S L S I T C T V S G F S
 CGATGGCCCCAGGTGCAGCTGCAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCATCACATGCACCGTCTCAGGGTTCT
 101 111 121 131 141 151 161 171 181

L T G Y G V N W V R Q P P G K G L E W L G M I W G D G N T D
 CATTAACCGGCTATGGTGTAAACTGGGTTCGCCAGCCTCCAGGAAAGGGTCTGGAGTGGCTGGGAATGATTTGGGGTGATGGAAACACAG
 191 201 211 221 231 241 251 261 271

Y N S A L K S R L S I S K D N S K S Q V F L K M N S L H T D
 ACTATAATTCAGCTCTCAAATCCAGACTGAGCATCAGCAAGGACAACCTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGTCTGCACACTG
 281 291 301 311 321 331 341 351 361

D T A R Y Y C A R E R D Y R L D Y W G Q G T T V T V S S A S
 ATGACACAGCCAGGTACTACTGTGCCAGAGAGAGATTATAGGCTTGACTACTGGGGCCAAGGCACCACGGTCACCGTCTCCTCAGCCT
 371 381 391 401 411 421 431 441 451

T K G P S V F P L A P S S K S T S G G T A A L G C L V K D Y
 CCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACT
 461 471 481 491 501 511 521 531 541

F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G L
 ACTTCCCCGAACCGGTGACGGTGTCTGTGGAACCTCAGGCGCCCTGACCAGCGGCGTGCACACCTTCCCGGCTGTCTACAGTCCTCAGGAC
 551 561 571 581 591 601 611 621 631

Fig. 10. cont. (1)

Y S L S S V V T V P S S S L G T Q T Y I C N V N H N P S N T
TCTACTCCCTCAGCAGCGTGGTGA CTGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAACCCAGCAACA
641 651 661 671 681 691 701 711 721

K V D K K V E P K S S * * M K
CCAAGGTCGACAAGAAAGTTGAGCCCCAAATCTTCATAATAACCCGGGAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAA
731 741 751 761 771 781 791 801 811

Y L L P T A A A G L L L P A A Q P A M A D I E L T Q S P A S
ATACCTATTGCCTACGGCAGCGCTGGATTGTTATTACCTGCTGCCCAACCAGCGATGGCCGACATCGAGCTCACCCAGTCTCCAGCCTC
821 831 841 851 861 871 881 891 901

L S A S V G E T V T I T C R A S G N I H N Y L A W Y Q Q K Q
CCTTTCTGCGTCTGTGGGAGAACTGTCACCATCACATGTCGAGCAAGTGGGAATATTCACAATTATTTAGCATGGTATCAGCAGAAACA
911 921 931 941 951 961 971 981 991

G K S P Q L L V Y Y T T T L A D G V P S R F S G S G S G T Q
GGGAAAATCTCCTCAGCTCCTGGTCTATTATACAACAACCTTAGCAGATGGTGTGCCATCAAGGTTCAAGTGGCAGTGGATCAGGAACACA
1001 1011 1021 1031 1041 1051 1061 1071 1081

Y S L K I N S L Q P E D F G S Y Y C Q H F W S T P R T F G G
ATATTCTCTCAAGATCAACAGCCTGCAGCCTGAAGATTTTGGGAGTTATTACTGTCAACATTTTGGAGTACTCCTCGGACGTTCCGGTGG
1091 1101 1111 1121 1131 1141 1151 1161 1171

G T K L E I K R T V A A P S V F I F P P S D E Q L K S G T A
AGGCACCAAGCTCGAGATCAAACGGACTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAAGTGC
1181 1191 1201 1211 1221 1231 1241 1251 1261

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Fig. 10. cont. (2).

S V V C L L N N F Y P R E A K V Q W K V D N A L Q S G N S Q
CTCTGTGTGTGTCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCA

1271 1281 1291 1301 1311 1321 1331 1341 1351

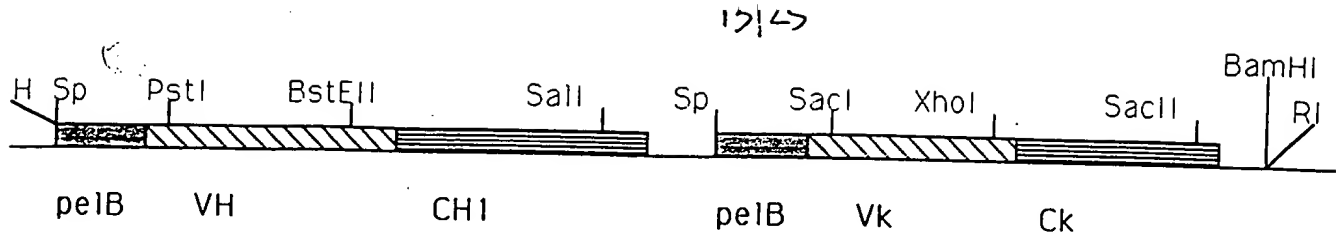
E S V T E Q D S K D S T Y S L S S T L T L S K A D Y E K H K
GGAGAGTGTACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAA

1361 1371 1381 1391 1401 1411 1421 1431 1441

V Y A C E V T H Q G L S S P V T K S F N R G E S * *
AGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGTTGCGCCGTCACAAAGAGCTTCAACCGCGGAGAGTCATAGTAAGGATCCAGCTC

1451 1461 1471 1481 1491 1501 1511 1521 1531

GAATTC



FabD1.3 in
pUC19

Fig. 10 cont. (3).

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Fig. 11 Comparison of lysozyme-binding by phage-Fab and phage -ScFv

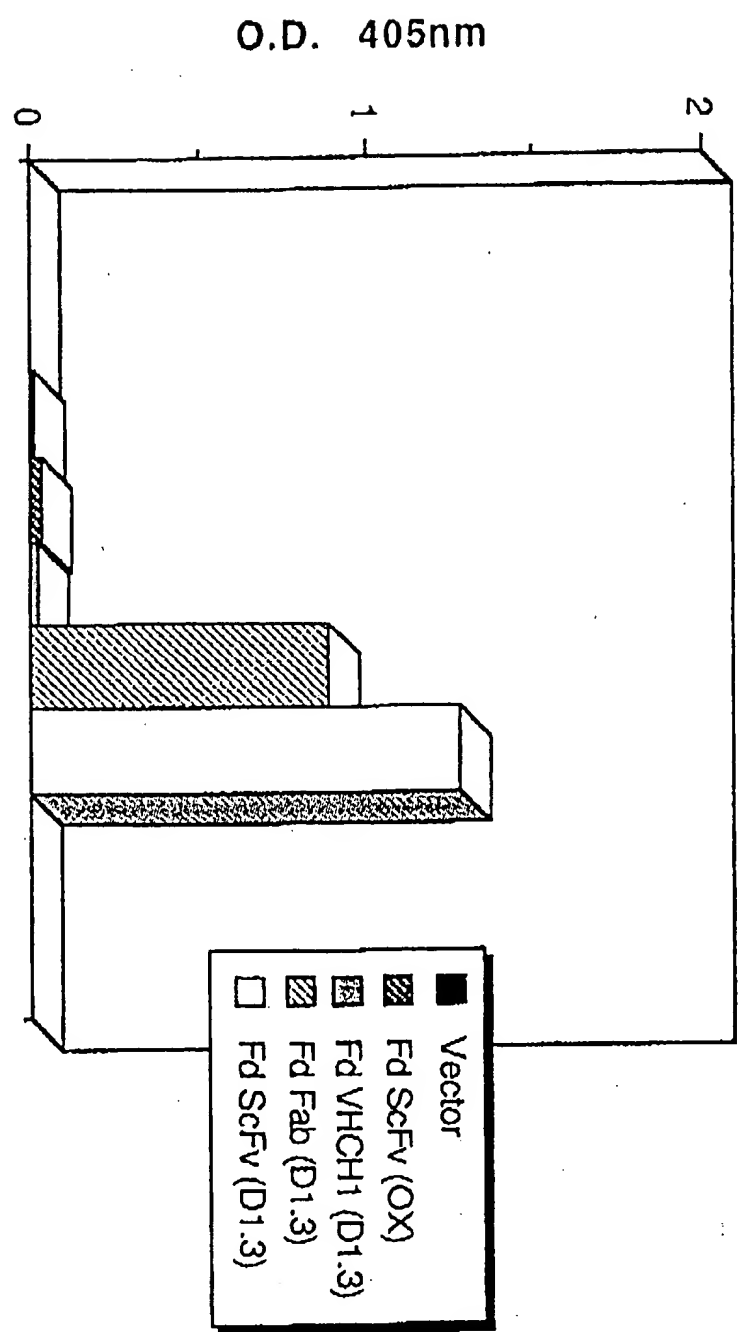


Fig. 12



Figure 13 Sequence of pNQ11ScFv

Q V Q L Q E S G G G L V Q P G G
 CAG GTG CAG CTG CAG GAG TCA GGA GGA GGC TTG GTA CAG CCT GGG GGT
 PstI
 S L R L S C A T S G F T F S N Y
 TCT CTG AGA CTC TCC TGT GCA ACT TCT GGG TTC ACC TTC AGT AAT TAC
 Y M G W V R Q P P G K A L E W L
 TAC ATG GGC TGG GTC CGC CAG CCT CCA GGA AAG GCA CTT GAG TGG TTG
 G S V R N K V N G Y T T E Y S A
 GGT TCT GTT AGA AAC AAA GTT AAT GGT TAC ACA ACA GAG TAC AGT GCA
 S V K G R F T I S R D N F Q S I
 TCT GTG AAG GGG CGG TTC ACC ATC TCC AGA GAT AAT TTC CAA AGC ATC
 L Y L Q I N T L R T E D S A T Y
 CTC TAT CTT CAA ATA AAC ACC CTG AGA ACT GAG GAC AGT GCC ACT TAT
 Y C A R G Y D Y G A W F A Y W G
 TAC TGT GCA AGA GGC TAT GAT TAC GGG GCC TGG TTT GCT TAC TGG GGC
 Q G T L V T v s s g g g g s g g g g s
 CAA GGG ACC CTG GTC ACC gtc tcc tca ggtggaggcgggttcaggcggaggtggctct
 BstEII
 g g g g s d i E L T Q T P L S L P V
 ggcgggtggcgatcggac atc GAG CTC ACC CAA ACT CCA CTC TCC CTG CCT GTC
 SacI
 S L G D Q A S I S C R S S Q S I
 AGT CTT GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC ATT
 V H S N G N T Y L E W Y L Q K P
 GTA CAT AGT AAT GGA AAC ACC TAT TTA GAA TGG TAC CTG CAG AAA CCA
 PstI
 G Q S P K L L I Y K V S N R F S
 GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT
 G V P D R F S G S G S G T D F T
 GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCG GGG ACA GAT TTC ACA
 L K I S R V E A E D L G V Y Y C
 CTC AAG ATC AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TAC TGC
 F Q G S H V P Y T F G G G T K L
 TTT CAA GGT TCA CAT GTT CCG TAC ACG TTC GGA GGG GGG ACC AAG CTC
 E I K R
GAG ATC AAA CGG
 XhoI

Restriction sites referred to in the text are shown underlined. The sequence contributed by the linker is shown in lower case.

Figure 14. Binding of pAbs to specific antigens

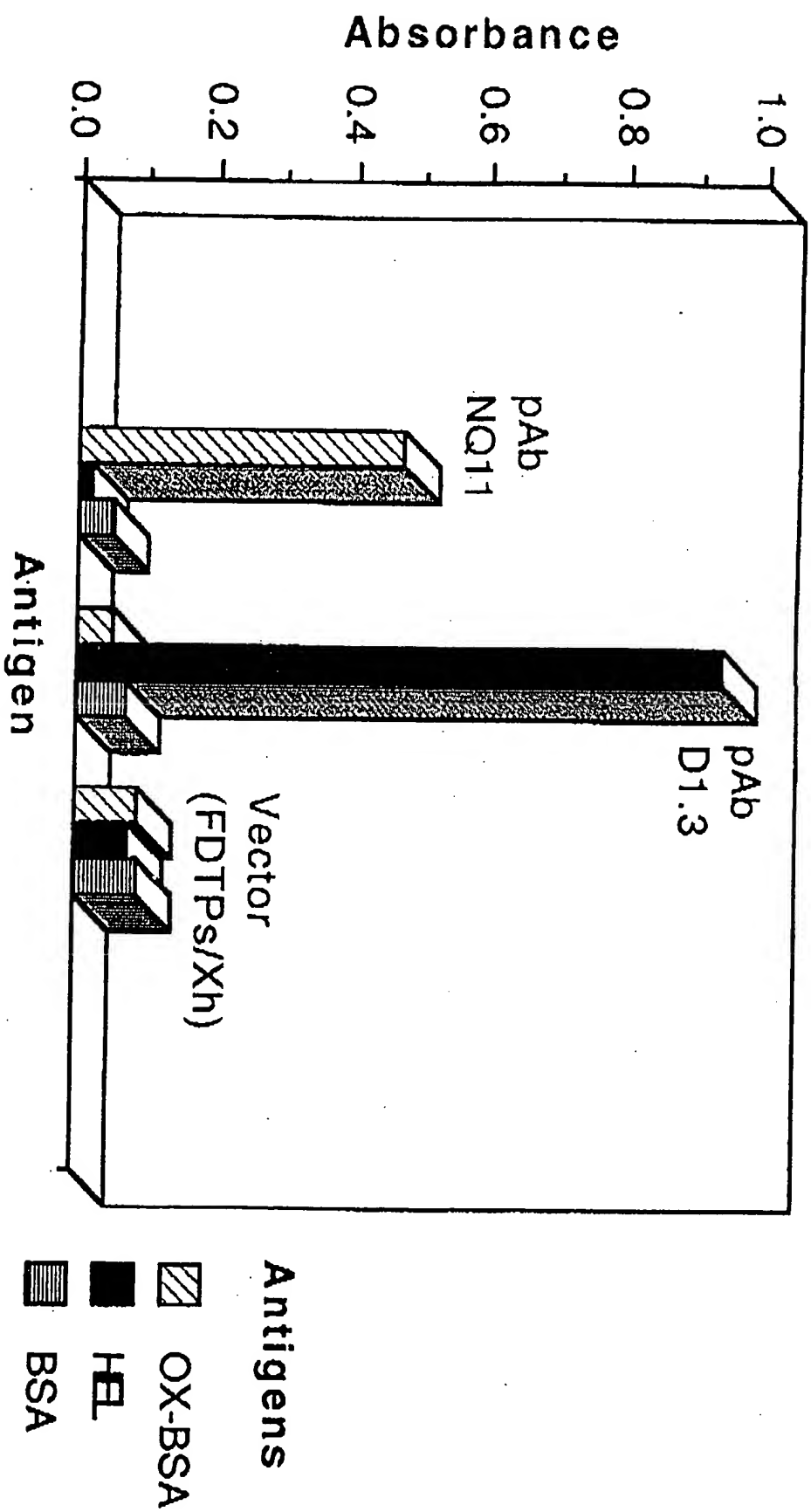


Fig.15. Sequence surrounding phoA insertion in fd-phoA1

SEQUENCE AT 5' END OF phoA INSERTION IN fd-phoA1

Signal peptide
cleavage site
▼
TCT CAC AGT GCA CAA ACT GTT GAA CGG ACA CCA GAA ATG CCT GTT CTG
R T P E M P V L
ApaLI

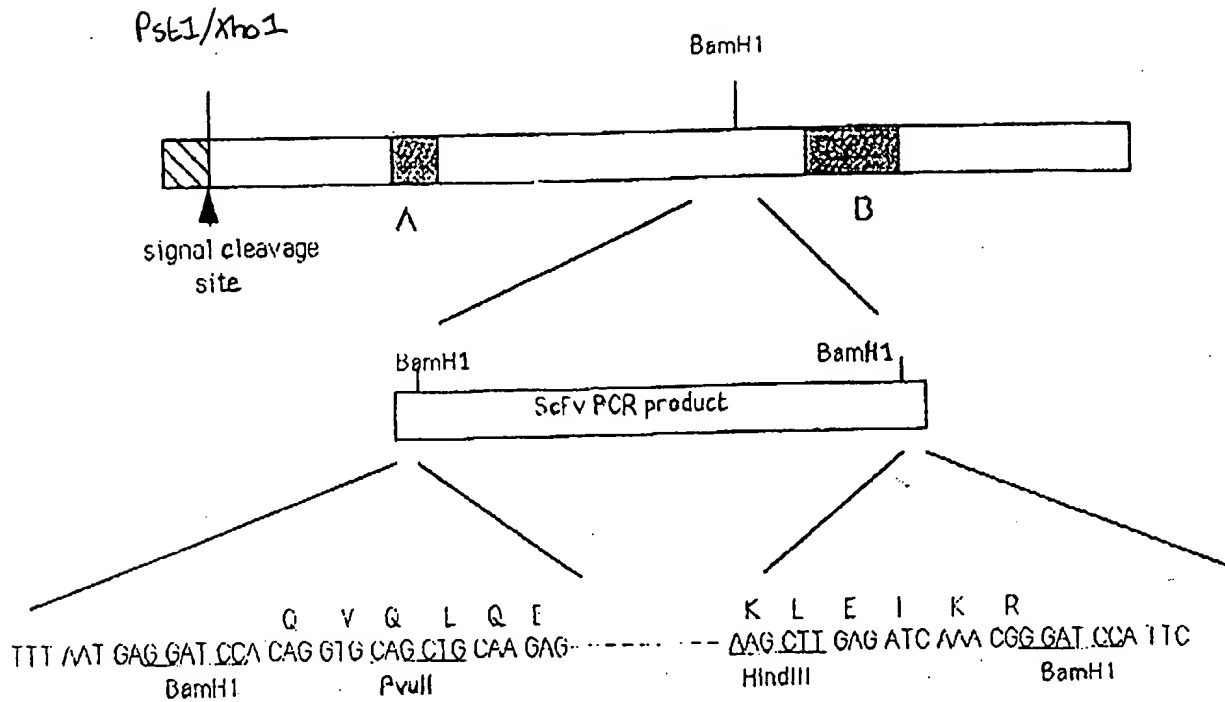
SEQUENCE AT 3' END OF phoA INSERTION IN fd-phoA1

K A A L G L K
AAA GCC GCT CTG GGG CTG AAA GCG GCC GCA GAA ACT GTT GAA AGT etc.
NotI

The restriction sites used for cloning are shown as well as the amino acids encoded by phoA around the insertion site. In this example, the first five amino acids of the mature fusion will actually come from gene 3.

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Figure 16. Structure of gene 3



2)

A

(1834) 5' GAG GGT GGT GGC TCT
 " " "C " "
 " " "C " "
 " " "C " ACT 3'(1839)

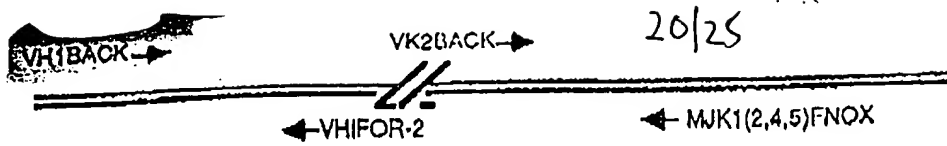
B

(2284) 5' - GGC GGC GGC TCT
 - GGT GGT GGT "
 - " GGC GGC "
 GAG " " GGC "
 " " " GGT "
 " " " GGC "
 " " " GGT "
 " " " GGC " 3'(2379)

Reverse complement of mutagenic
 oligo G3BamIink

5' GAG GGT GGC GGA TCC
 T
 GAG GGT GGC GG 3'

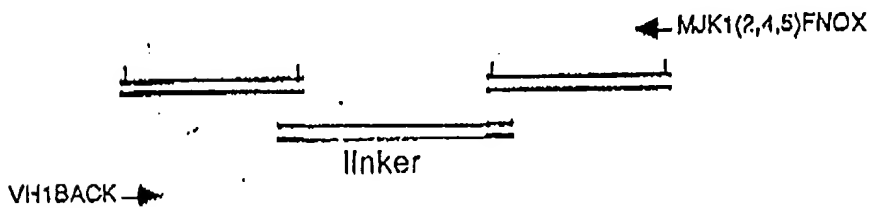
Numbering is according to Beck et al (1978, supra).



PRIMARY PCR FROM cDNA

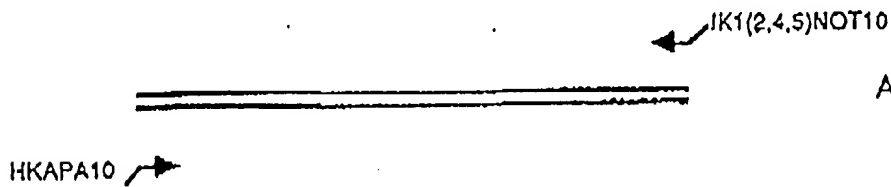


MIX PRODUCTS WITH LINKER DNA
(MADE BY PCR)



Primary PCR
products

SECOND ASSEMBLY PCR
USING 'OUTER' PRIMERS



Assembled combinatorial
product

PULL-THROUGH (tagged primers)



DIGEST AND CLONE

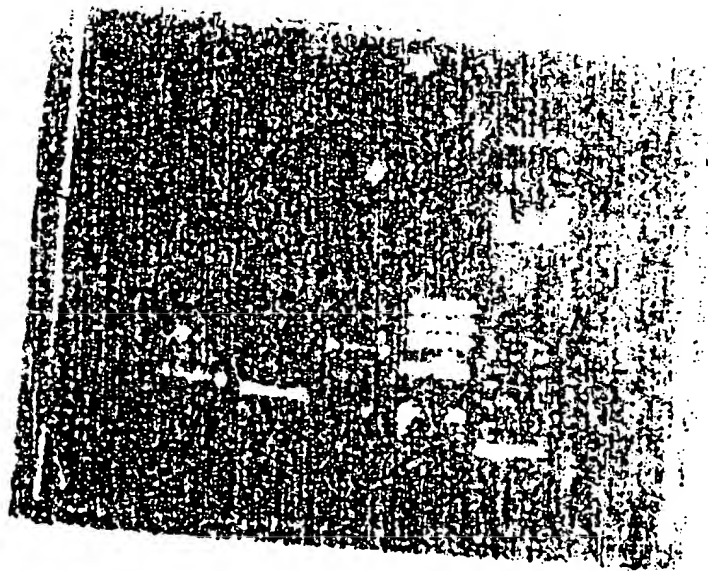
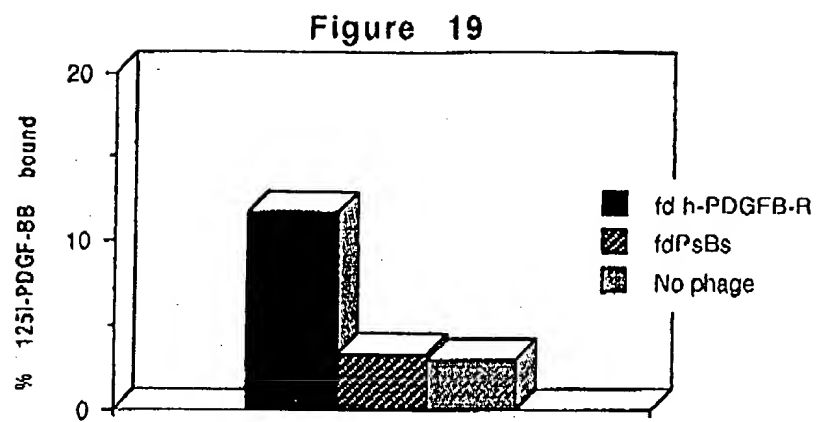


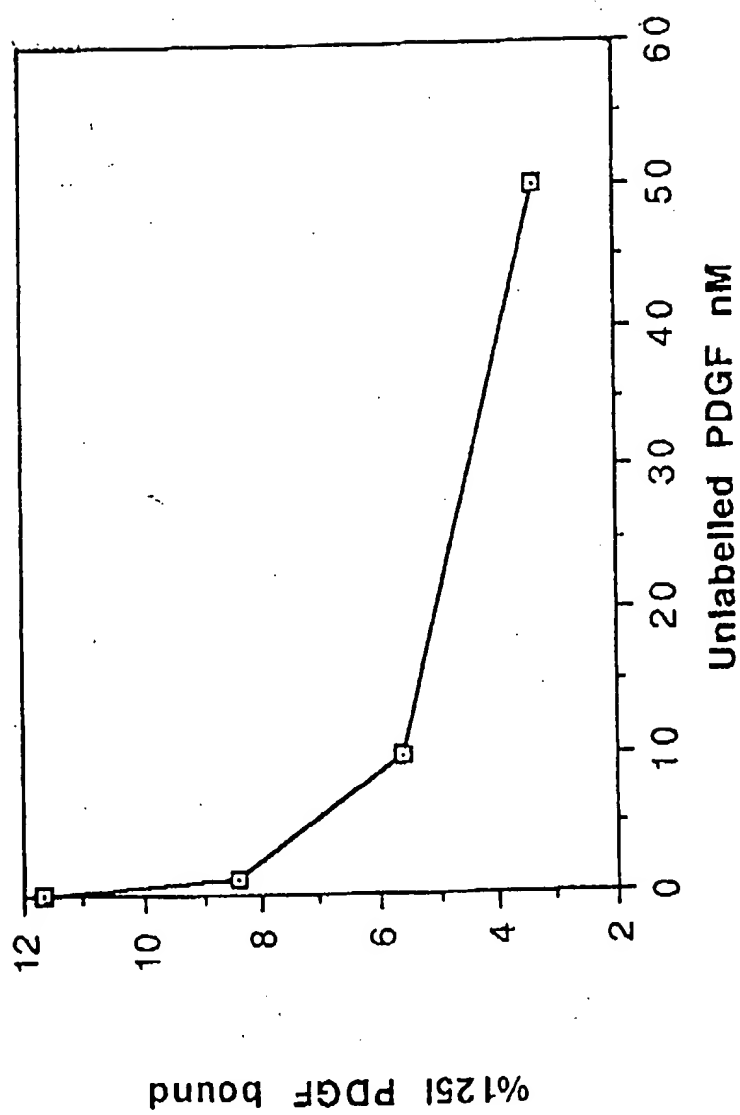
Fig. 18

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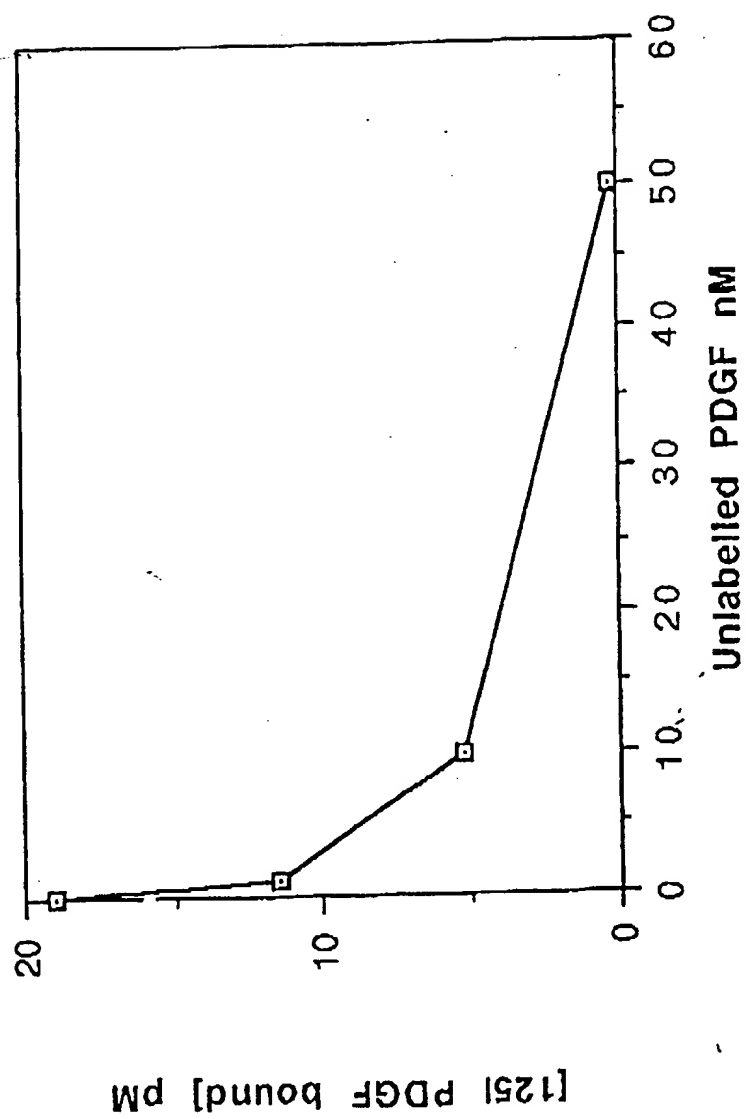
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Figure 20 Displacement by unlabelled PDGF



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Figure 21 PGDF displacement vector binding deducted



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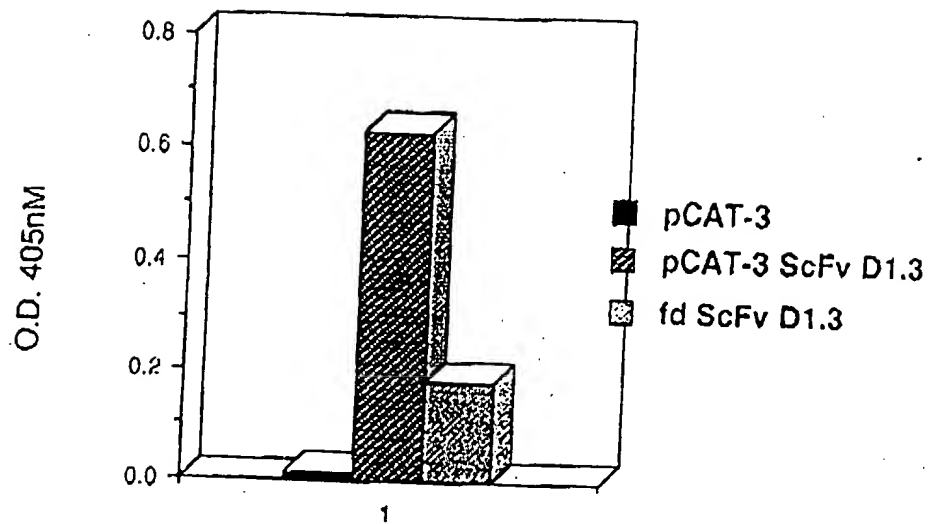


Figure 22: Elisa of lysozyme binding by pCAT-3 ScFv D1.3 phagemid in comparison with pCAT-3 vector (both rescued by M13KO7) and fdCAT2 ScFvD1.3 as described in example 17. ELISA was performed as described in example 6 with modifications detailed in example 17.

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